Fraunhofer-Gesellschaft zur Förderung
der angewandten Forschung e.V.
Our Ref.: C 2428 PCT

Molecular Pathogenicide Mediated Plant Disease Resistance

FIELD OF THE INVENTION

The present invention relates to gene constructs suitable for expressing agents to protect a plant against pathogens and the suitable proteins for such plant protection. These agents are named "molecular pathogenicides". This invention is related to the genetic engineering of plants and to means and methods for conferring pathogen resistance on a plant using a gene or genes encoding: a pathogen specific antibody and a pathogen specific antibody including a toxic activity which blocks stages of the pathogen life cycle, pathogen replication or pathogen movement within a plant or pathogen transmission from plant to plant. The means and methods are given for soluble expression of recombinant antibodies, antibody fusion proteins and antibody protein complexes in different plant cell compartments or the immobilisation of recombinant antibodies, antibody fusion proteins and antibody protein complexes in cellular membranes in different orientations and the display of recombinant proteins on the plant cell membrane. This invention also describes novel methods and protein binding partners for assembling protein complexes from individual polypeptide chains during expression of these proteins in vivo. Also given are the methods for activation of the molecular pathogenicides by in vivo proteolytic cleavage.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturers specifications, instructions, etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art of the present invention.

BACKGROUND OF THE INVENTION

Plant disease constitutes a major and ongoing threat to human food stocks and animal feed. Most crop plants are regularly exposed to one or more pathogen(s) that

can cause incredible damage resulting in substantial economical losses every year. Attack by pathogens, such as viruses, bacteria, fungi, nematodes and insects and is a severe economic problem, which impacts all economically important crops, for example rice, soybean, sweet potato, wheat, potato, grape, maize and ornamental plants. Current protective measures rely heavily on chemical control measures for pathogen vectors, which have undesirable environmental consequences.

A more effective approach to protecting plants from pathogen attack is to create plants that are endogenously resistant to pathogens. However, plant breeders have limited sources of resistance genes against plant diseases. This can now be achieved using genetic engineering techniques, by providing the plant with genetic information required for affecting the pathogens and for being resistant to the disease caused by the pathogen. For example, in the case of a viral pathogen, the host plant is resistant if it has the ability to inhibit or retard the replication of a virus, the symptoms of viral infection or the life cycle of the virus, including its transmission. "Resistant" is the opposite of "susceptible" and may be divided into three levels:

1) Full,

2) Medium,

3) Partial resistance,

A plant may be considered fully resistant when it shows no symptoms on infection and there is no evidence of pathogen replication or reproduction. The host plant may be resistant to the establishment of infection, pathogen reproduction and/or pathogen movement and transmission.

In recent years, the advances in plant molecular virology have enhanced the understanding of pathogen genome organisation and gene function. Moreover, genetic engineering of plants for virus resistance has recently provided new strategies for control of viral disease (Baulcombe, 1994), (Gadani et al., 1990), (Wilson, 1993). The following genes were expressed in transgenic plants in order to confer resistance: viral coat proteins, non-structural proteins of viral genomes, viral anti-sense transcripts, viral satellite RNAs, ribozymes and interferon genes (Baulcombe, 1994), (Gadani et al., 1990), (Wilson, 1993), (Harrison et al., 1987), (Namba et al., 1991), (Anderson et al., 1992). Although most of these approaches have been effective for attenuating infections, resistance was not complete and confined to a small spectrum of viral pathogens (Falk and Bruening, 1994), (Wilson, 1993) and bears significant risks (Palukaitis and Roossinck, 1996).

The major disadvantages of these methods are:

1) Host range is limited.
2) Pathogen range is limited.
3) Resistance is partial and though symptoms are delayed infection still results in the disease.
4) Resistance could be broken in case of coat protein mediated resistance and ribozyme mediated resistance.
5) Expression of viral proteins can lead to enhanced pathogen activity. For example, in the case of viral coat protein mediated resistance, cross encapsidation of mild non-pathogenic strains of virus by the expressed coat protein can occur which then leads to development of a more severe disease.

An alternative way to protect plants against pathogen infection is the generation and expression of recombinant antibodies (rAbs), which are often referred to as **"Plantibodies"**. Pathogen-specific recombinant antibodies targeted to different compartments of plant cells or different plant organs overcome many of the problems mentioned before and confer a broader spectrum of resistance to disease (Baulcombe, 1994). To achieve this, recombinant antibodies (Plückthun, 1991), (Winter and Milstein, 1991) against the target proteins have to be generated by cloning the corresponding antibody heavy and light chain genes from hybridoma cells, synthetic, semi-synthetic and immunocompetent phage display or ribosome display libraries; or by the generation of fully synthetic designer antibodies. This is followed by subsequent modification and rAb expression in different compartments of heterologous hosts such as bacteria, yeast, algae, baculovirus infected insect cells, mammalian cells and plants. For example, antibodies and antibody-fusion proteins binding to conserved functional domains of viral coat proteins, movement proteins, replicases or transmission factors can be used to inactivate such targets inside or outside the plant cell through immunomodulation. The feasibility of expressing recombinant antibodies (Plückthun, 1991), (Winter and Milstein, 1991) for the generation of resistance has been shown recently for both animal (Chen et al., 1994), (Duan et al., 1994), (Marasco et al., 1993) and plant viruses (Tavladoraki et al., 1993), (Voss et al., 1995), (Zimmermann et al., 1998). Single chain antibody fragments derived from monoclonal antibodies (scFvs) (Bird et al., 1988) directed against *Rev* (Duan et al., 1994) and gp120 (Chen et al., 1994) (Marasco et al., 1993)

of HIV, inhibited HIV-replication, virion assembly and syncytia formation when expressed intracellularly, or within the ER of human cells.

Interestingly, intracellular expression of an scFv specific for the artichoke mottled crinkle virus coat protein in transgenic Tobacco caused a reduction of infection and a delay in symptom development (Tavladoraki et al., 1993). Targeting of TMV-specific full-size antibodies to the intercellular space of Tobacco plants inhibited viral infections up to 70% (Voss et al., 1995). In the latter case, plant produced antibodies showed the same specificity and affinity for TMV (Fischer et al., 1998) as the parental murine antibody. Cytosolic expression of an engineered scFv derived from this anti-TMV antibody yielded fully resistant Tobacco plants, even under systemic infection conditions (Zimmermann et al., 1998). These studies demonstrate the potential of heterologously expressed recombinant antibodies to combat pathogens via intra- or extra-cellular modulation of pathogen proteins.

Plant cells can synthesise large amounts of antibodies that are functionally indistinguishable from the source monoclonal. For example, full-size antibodies (Düring et al., 1990), (Hiatt et al., 1989), (Voss et al., 1995), Fab-fragments (De Neve et al., 1993), scFvs (Owen et al., 1992; Zimmermann et al., 1998), (Tavladoraki et al., 1993), scFv fusion proteins (Spiegel et al., Plant Science 149 (1999), 63-71), bispecific scFv (Fischer et al., 1999) and dAbs (Benvenuto et al., 1991) have been successfully expressed in Tobacco, Potato (Schouten et al., 1997) or *Arabidopsis*, reaching expression levels as high as 6.8% of the total protein (Fiedler et al., 1997).

Targeting of recombinant antibodies by exploiting known protein trafficking signal sequences now permits rAb expression in the cytoplasm (scFv fragments (Tavladoraki et al., 1993; Zimmermann et al., 1998)), the endoplasmic reticulum (Fiedler et al., 1997), chloroplasts (Düring et al., 1990) and the intercellular space (Benvenuto et al., 1991; De Neve et al., 1993; Voss et al., 1995; Zimmermann et al., 1998) (full-size, Fab fragments, scFvs and single domain Abs). These results demonstrate the flexibility of the plant system to express any recombinant antibody or recombinant antibody fragments in almost all plant compartments, using targeting sequences that also may be from plants or derived from other eukaryotes.

The advantage of targeted protein expression is that the rAbs can be expressed where the pathogen is most vulnerable and where they will have the maximal protective effect. In patent application WO 96/09398 the use of antibody-fusion proteins as agents for controlling crop disease caused by pathogens is proposed.

The antibody delivers a toxin which kills the pathogen in transgenic plants or when expressed or applied as an external immunotoxin. WO 96/09398 is focussed on recombinant Ab-fusion proteins – single polypeptides that are either genetically, chemically or "biochemically" linked to form an immunotoxin. However, WO 96/09398 does not provide proof of principle for antibody mediated pathogen resistance and it was doubtful whether any of the hypothetical examples in WO 96/09398 would work to the extent that a protection of plants against pathogen attack can be obtained sufficient to comply with the needs of the breeders and farmers. Thus, there is still a need of means and methods for conferring antipathogenic/predator characteristics to transgenic plants.

SUMMARY OF THE INVENTION

The objective of this current patent application is to provide means and methods for protecting plants, in particular monocotyledonous and dicotyledonous agricultural crops and ornamental plants, against pathogens in a more effective and environmentally sensitive manner. Accordingly, the solution to the technical problem is achieved by providing the embodiments characterised in the claims.

As will be described hereinbelow, the above-mentioned objective is met according to the invention by any one of the following or any combination of the following inventions: i) the expression of pathogen specific recombinant antibodies and parts thereof, or ii) by fusing antibodies or parts thereof to toxins, proteins, or enzymes having activity against the pathogens or to the effective parts of these toxins or enzymes, and then expressing these fusion proteins, or iii) by assembling protein complexes composed of an antibody or fragment thereof *in vivo* using the novel binding proteins described here and or iv) including a specific protease sensitive sequence, that is cleaved (e.g. in the presence of the pathogen or in a specific plant cell compartment) to release and or activate the toxic activity of any of the recombinant proteins in i) to iii), and or v) targeting or integrating any of the recombinant proteins in i) to iv) to cell membranes in any orientation. These agents are also named "molecular pathogenicides". Thus, in one aspect the present invention relates to a fusion protein comprising

(a) at least one binding domain specifically recognising an epitope of a plant pathogen; and

(b) at least one further domain comprising a protein or peptide sequence which is toxic to the pathogen or detrimental to its replication, transmission or life cycle.

Said domains can be linked by covalent or non-covalent bonds. In a preferred embodiment of the fusion protein of the invention said binding domain comprises an antibody, a T-cell receptor, a pathogen specific receptor, a peptide specific for an epitope of a pathogen, or at least the binding site of any one of those.

In another aspect, the invention relates to membrane associated binding domains and further domains, respectively, as defined herein.

The fusion proteins composed of a pathogen specific antibody and toxin molecule can be made by fusing the respective parts by genetic or biochemical means. In addition, the chimeric protein can preferably be assembled *in vivo* from its parts by the plant or via expression in the organisms' endogenous protein machinery. In a particularly preferred and advantageous embodiment of the invention, these domains or parts thereof, fusion proteins or protein complexes can also be targeted to organelles and plant cell compartments or immobilised and membrane anchored by the addition of signal sequences and or membrane anchors. The recombinant molecular pathogenicide protein preferably contains specific protease cleavage sequences that are cleaved *in vivo,* by a plant and/or a pathogen specific protease(s), to release and or activate the toxic agent(s), or parts thereof, upon infection.

The fusion protein of the present invention can further comprise a carrier protein suitable for delivering the fusion protein or its domains into a host cell, preferably plant cell or a cellular compartment thereof. Furthermore, the fusion protein of the present invention can comprise a fluorophore such as green fluorescent protein fused to at least one of the above-described domains the fusion protein consists of.

In a further aspect, the present invention relates to a pathogenicide comprising at least one binding and/or further domain as defined herein and a cellular targeting sequence and/or membrane localisation sequence and/or motif that leads to membrane anchoring. Preferably, the membrane localisation sequence is proteolytically sensitive.

Suitable membrane anchor sequences, enabling the integration of secretory recombinant antibody fusion proteins and parts thereof in the plasma membrane, include the human T cell receptor transmembrane domains (Gross and Eshhar, 1992), glyco-phosphatidyl inositol (GPI) anchors (Gerber et al., 1992), immunoglobulin superfamily membrane anchors, tetraspan family members (Tedder and Engel, 1994; Wright and Tomlinson, 1994) and any transmembrane sequence(s) from a known protein or synthesised sequences that have a similar function and can be included in the target protein by recombinant DNA technology. Fusion of a protein to these sequences would permit display of the recombinant protein on the lumenal face of organelles of the secretory or endocytic pathway or the plant cell membrane. This has the advantage that the recombinant protein can be targeted to the intracellular space where many pathogens are most vulnerable.

In addition, the antibodies or parts thereof, or the recombinant antibody fusion proteins, or parts thereof, may be targeted to cell membranes where they could face the cytosolic side of the membrane. Suitable targeting sequences for cytoplasmic display, include the transmembrane domains of: KAR1, for nuclear membrane integration (Rose and Fink, 1987), middle-T antigen (Kim et al., 1997), for plasma membrane integration and cytochrome b5, for ER membrane integration (Kim et al., 1997). C-terminal linkages to fatty acids using consensus amino acid sequences leading to post translational prenylation, farnesylation, palmitoylation, myristoylation or ankyrin sequence motifs can also be used. This cytoplasmic display method has the significant advantage that the recombinant proteins can be localised at the site of intracellular pathogen replication, where they will have the most potent effect. In addition, membrane localisation of proteins stabilises the protein and reduces the effect of C-terminal protein degradation *in vivo*. Preferably, the pathogenicide of the invention comprises the fusion protein described herein.

In a particularly preferred embodiment, the present invention relates to the described pathogenicides wherein said binding domain(s) and/or said further domain(s) are capable of self assembly in vivo.

In a further embodiment, the present invention relates to a polynucleotide encoding a fusion protein or pathogenicide of the invention. Thus, the invention relates to one or

more gene constructs that encode a nucleotide sequence encoding an antibody or part thereof which is specific for a pathogen and in the case of fusion proteins, for a nucleotide sequence encoding a protein, enzyme or peptide which has detrimental effects on a pathogen and ideally is toxic to the pathogen. This invention includes antibodies specific for the pathogen and/or for host proteins utilised by the pathogen during its life cycle. This invention also relates to chimeric proteins that consist of an antibody, antibodies or parts thereof, which are specific for a pathogen, and a protein or peptide which has detrimental or ideally toxic effects on the pathogen and which has been constructed by biochemically linking the antibody or parts thereof to the toxin. Furthermore, the present invention relates to a vector comprising the polynucleotide of the invention. Said vector can comprise separate polynucleotides encoding at least one of said binding domain(s) and/or said further domain(s) of the above-described fusion protein. In addition, the present invention relates to a composition comprising vectors wherein each vector contains at least one polynucleotide encoding at least one binding domain and/or at least one further domain of the fusion protein or the pathogenicide of the invention; and wherein the expression of at least two of said polynucleotides results in the production of said fusion protein or said pathogenicide or assembly of the same in vivo.

In a preferred embodiment of the vector or the composition of the invention the polynucleotide is operatively linked to regulatory sequences allowing the expression of the fusion protein, pathogenicide or the domains thereof in a host cell. Said regulatory sequence can be a constitutive, chimeric, tissue specific or inducible promoter.

Furthermore, the present invention relates to a host cell comprising any one of the above-described polynucleotides, vectors or vectors of the compositions.

In another embodiment the present invention relates to a method for the production of a molecular pathogenicide comprising:

(a) culturing the host cell of the invention under conditions suitable for the expression of the polynucleotide; and

(b) recovering the fusion protein, pathogenicide or the domains thereof from the culture.

The present invention also relates to a molecular pathogenicide obtainable by the method of the invention or encodable by the polynucleotide of the invention.

This invention also relates to *in vivo* assembled protein complexes composed of one or more discrete polypeptide chains, encoded by separate nucleotide sequences on one or more constructs, that are assembled by the plant or expression organisms protein synthesis machinery into a protein complex.

Furthermore, the present invention relates to a method for the production of pathogen resistant transgenic plants, plant cells or plant tissue comprising the introduction of a polynucleotide or vector of the invention or the vectors of the composition of the invention into the genome of a plant, plant cell or plant tissue.

The present invention also relates to a transgenic plant cell which contains stably integrated into the genome a polynucleotide or vector of the invention or the vectors of the composition of the invention or obtainable according to the method of the invention.

In addition, the present invention relates to a transgenic plant or plant tissue comprising the above-described plant cells or obtainable by the method of the invention. Encompassed are also the transgenic plants wherein the fusion protein or pathogenicide are made functional against pathogens by *in vivo* assembly after co-transformation of at least two independent plant expression constructs or after sexual crossing to form hybrid offspring from two parental plants expressing one or more of the domains of the fusion protein or the pathogenicide, or any other form of genetic recombination. Preferably, the transgenic plant of the invention displays improved resistance against a pathogen that the wild type plant was susceptible to.

Furthermore, the present invention relates to harvestable parts and propagation material of a plant of the invention comprising plant cells of the invention.

In a still further embodiment, the present invention relates to a kit comprising any one of the described fusion proteins, pathogenicides, polynucleotides, compositions or molecular pathogenicides of the invention.

In another embodiment the present invention relates to the use of the described antibodies, fusion proteins, polynucleotides, vectors, compositions and molecular pathogenicides of the invention in agriculture for the protection of a plant against the action of a pathogen.

Some aspects of the present invention will be described herein below in more detail.

The term "binding domain" is used to denote polypeptide chain(s) which exhibit a strong monovalent, bivalent or polyvalent binding to a given epitope or epitopes. Preferably, said binding domain is an antibody or a binding site thereof. The antibodies may be generated by hybridoma technology, or ribosome display, or phage display, of natural naïve origin, or immunised origin, semi-synthetic or fully synthetic libraries. The term "antibody" is also used to denote designer antibodies. These antibody polypeptides are encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind the given epitope or epitopes. The recognised immunoglobulin genes include the kappa and lambda light chain genes, the mu, delta, gamma, alpha and epsilon constant regions as well as all immunoglobulin variable regions from vertebrate, camelid, avian and pisces species. The term antibody, as used herein, includes in particular those antibodies synthesised or constructed *de novo* using recombinant DNA methodology, such as recombinant full-size antibodies, dimeric secretory IgA antibodies, multimeric IgM antibodies, $F(ab')_2$-fragments, Fab-fragments, Fv-fragments, single chain Fv-fragments (scFvs), bispecific scFvs, diabodies, single domain antibodies (dAb), minibodies and molecular recognition units (MRUs). Antibody sequences may be derived from any vertebrate, camelid, avian or pisces species using recombinant DNA technology, or also by using synthetic, semi-synthetic and naïve or immunocompetent phage and ribosome display libraries, gene shuffling libraries, and fully synthetic designer antibodies. In this invention, the antibodies are generated against specific pathogen or host plant epitopes that are involved in the pathogen replication, reproduction or life cycle.

The term "pathogen" is used to denote viral or virus like organisms, bacteria, mycoplasmas, fungi, insects or nematodes that affect the germination of seed, growth, development, reproduction, harvest, yield or utility of a plant.
The term "toxic" refers to an activity, which may be peptide or polypeptide encoded, that affects the reproduction or replication of a pathogen and/or any stages of its life cycle. In the case of viral pathogens, this includes entry into the plant, viral uncoating and disassembly, viral replication, viral assembly, cell to cell and long distance movement and the development, spread, or life cycle of the virus. Suitable toxic activities include RNAse (Leland et al., 1998) and DNAse, ribosome inactivating proteins (Barbieri et al., 1993), (Girbes et al., 1996), (Hartley et al., 1996) and or toxins with antimicrobial activity (Dempsey et al., 1998). Antibodies or recombinant proteins in themselves are also considered toxic when they affect the pathogen by binding to pathogen and or host proteins that are utilised by a pathogen during its replication, reproduction, life cycle or transmission. For example, a fusion protein composed of a virus specific antibody and a viral coat protein will interfere with virus reproduction by both binding to the virus and by disrupting viral assembly or disassembly in the host cell.

The term "molecular pathogenicide" refers to the antibodies and proteins described in this application, which have toxic effects on pathogen(s) either as single fusion proteins, when expressed in combination with other proteins, or when expressed as part of protein complexes that are assembled *in vivo*.

Monoclonal antibodies (Köhler and Milstein, 1975) can be raised against almost any epitope or molecular structure of a pathogen or host protein using several techniques. The most common method is the hybridoma technique starting with immunocompetent B lymphocytes from the spleen or thymus which are obtained after immunisation with native antigen, recombinant antigen, antigen fusion proteins, antigen domains or by *in vitro* or genetic immunisation. In addition, recent advances in molecular biology techniques now permit the use of cloned recombinant antibody fragments and antibodies derived from mice and other organisms than the mouse. Suitable recombinant antibody fragment(s) include the complete recombinant full-size antibodies, dimeric secretory IgA antibodies, multimeric IgM antibodies, the $F(ab')_2$ fragment, the Fab-fragment, the Fv-fragment, single chain antibody fragments

(scFvs), single binding domains (dAbs), a bivalent scFv (diabody) (Poljak, 1994), minibody (Carter and Merchant, 1997), bispecific scFv antibodies (Plückthun and Pack, 1997) where the antibody molecule recognises two different epitopes, (which may be from the pathogen or the host or both the pathogen and the host), triabodies and any other part of the antibody such as, molecular recognition units (MRUs), which show binding to the target epitopes. Genes encoding these suitable recombinant antibody fragment(s) may be derived from vertebrates, camelids, avian or pisces species.

Also, single chain antibodies that have affinities for pathogen or host structures and proteins can be identified using phage display libraries or ribosome display libraries, gene shuffled libraries, which can be constructed from synthetic, semi-synthetic or naïve and immunocompetent sources (Plückthun, 1991; Winter et al., 1994; Winter and Milstein, 1991). Phage display and suitable techniques can be used to specifically identify antibodies, or fragments thereof, with the desired binding properties. Using recombinant antibody technology it is possible to identify antibodies or fragments that are highly specific for a single pathogen, or which recognise a consensus epitope conserved between several pathogens, where the antibodies will have a broad specificity against pathogens. The durability and effect of antibody mediated resistance can be improved by i) recombinant antibody affinity maturation, ii) CDR randomisation and selection, iii) stabilisation by framework optimisation of a selected pathogen specific antibody, iv) bi-specific antibody expression, v) the generation of antibody fusion proteins, or vi) the expression of antibodies in combinations with others that may potentiate their individual effects. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage displayed antibodies selections, yielding a high increment of affinity from a single library of phage antibodies which bind to an epitope of a pathogen (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13). The recombinant antibodies can be identified and utilised according to methods that are familiar to anyone of ordinary skill in the art.

Antibodies

This invention describes antibodies or fragments thereof which recognise structures of the pathogen or host plant and directly or indirectly lead to resistance or partial

resistance when expressed alone or when expressed as chimeric fusion protein coupled to a toxic activity or when expressed and assembled *in vivo* with a toxic activity to form an *in vivo* assembled molecular pathogenicide protein complex.
Antibodies can be generated that recognise pathogen-specific epitopes or host plant-specific epitopes which have a role in the life cycle of a pathogen. Suitable antibodies for engineering viral resistance include, but are not limited to, those binding to conserved functional domains of viral coat proteins, movement proteins, or replicases and are an approach to obtain broad-spectrum resistance and reduce the environmental risks by inactivating the targets inside and/or outside the plant cell through immunomodulation. The feasibility of this approach has been recently shown for both animal (Chen et al., 1994), (Duan et al., 1994), (Marasco et al., 1993) and plant viral resistance (Tavladoraki et al., 1993), (Voss et al., 1995), (Zimmermann et al., 1998). These antibodies or fragments thereof may be inactivating in themselves or in combination with one or more other antibodies, or a toxin, or in combination with a carrier, transmembrane domain or signal peptide. Importantly, plant pathogen resistance can be enhanced by the co-expression of multiple antibodies.

In a particular preferred embodiment, the present invention relates to one of the above-described antibodies wherein the antibody or a derivative thereof is capable of binding to the functional domain of a viral movement and/or replicase protein. As could be surprisingly demonstrated in Examples 5 and 6, antibodies directed against a viral movement and replicase, respectively, can be used to engineer enhanced resistance against the virus the movement and replicase gene are derived from. The advantage of using the movement or replicase protein as a target for the antibody or a functional equivalent binding protein is that the functional domains within the movement protein and the replicase can be expected to be highly conserved among different viruses. Thus, the expression of an antibody directed against such a conserved epitope of, for example, the movement protein of TMV can also be expected to be effective against related viruses. Furthermore, due to the conservation of the functional domains in these two viral proteins, a further advantage is that the heterogeneity within one single virus group should not be as high as for, e.g., the coat protein. Thus, the finding of the present invention that the movement and replicase protein of a virus are accessible to antibody targeting within a plant cell, a novel concept for the generation of virus resistant plants became

feasible. It is therefore, that in one separate aspect the present invention relates to such antibodies for engineering virus resistance in plants. Viruses that can be the target of this approach are any that use movement proteins during infection as well as all viruses that encode a replicase gene. This can be expected to be effective because viral movement is a common feature of many viral infections (McLean et al., Trends Microbiol. 1, (1993), 105-9) and replicases are essential for viral pathogenesis. The importance of approaches targeting these proteins is underscored by the fact that expressing wild type or defective versions of movement or replicase proteins often results in resistance (Beachy, (1997), Curr. Opin. Biotechnol. 8:215-220). Transgenic plants expressing defective mutant TMV movement protein are resistant to multiple viruses, presumably because of disruptions in intercellular viral movement (Cooper et al., (1995), Virology 206, 307-313) and replicase expression is an effective resistance strategy (Anderson et al., (1992), Proc. Natl. Acad. Sci. USA 89:8759-8763; Baulcombe, (1994), Trends Microbiol. 2:60-63; Brederode et al., (1995), Virology 207:467-474; Nguyen et al., (1996), Proc. Natl. Acad. Sci. USA 93:12643-12467; Rubino and Russo, (1995), Virology 212:240-243).

A disadvantage of the current antibody mediated resistance approaches may be the choice of viral coat proteins as target. Plant viral coat proteins have a broad structural diversity and this can restrict the effect of the expressed antibodies to a small range of viruses and under selective stress, the viral coat protein sequence can alter without loss of function. Generation of recombinant antibodies directed against conserved functional domains of viral replicases and movement proteins may provide a better route for obtaining pathogen resistant plants with a broad-spectrum resistance against viruses. The antigen for producing any one of the above-described antibodies can be derived from naturally occurring movement or replicase proteins or fragments thereof or can be recombinantly produced, chemically synthesized and/or derivatized by methods well known to the person skilled in the art some of which are also further discussed herein. In view of the above, the invention also relates to polynucleotides encoding the above-described antibodies, vectors comprising the same and host cells transformed therewith. Suitable vectors, host cells and strategies for the expression of recombinant antibodies in plants are described herein and can be easily adapted from any one of the other embodiments described herein.

Toxins

Toxins include all proteins and peptides that have a detrimental or toxic effect on a pathogen during its life cycle and/or an effect on the pathogen during plant infection or pathogen replication, spread or transmission. This includes toxins that specifically kill an infected host cell and so limit the spread and development of a disease.
Suitable toxins include the following:

- toxic peptide(s) which are specific for the pathogen and mediates toxicity e.g. by membrane permeabilisation based on alteration of membrane potential (Ham et al., 1994; Sangster, 1997).
- blocking peptides which bind to structural or non structural pathogen proteins, or nucleic acid motifs, and inhibit pathogen function, growth, development or toxicity to the host (Hayakawa, 1991; Silburn et al., 1998).
- peptide mimics that bind to pathogen or host protein motifs and that modulate or block the pathogen's replication, e.g. peptide derivatives of proteinase inhibitors that play a physiological role as inhibitors of viral replication and can be used as antiviral agents (Bjorck et al., 1990), (Bjorck et al., 1989).
- binding domains, such as antibodies defined above specifically recognising an epitope of a plant pathogen.
- peptide mimics that bind to pathogen or host protein motifs and that modulate or block the pathogen's movement within the host plant. As an example, the BC peptide, which mimics the nuclear localisation signal region of HIV-1, reduces HIV-1 production by 75% when expressed in infected dividing cultured human T-cells (Friedler et al., 1998).
- toxins which kill the host cell where the pathogen is replicating and has penetrated the cytosol (Barbieri et al., 1993; Hartley et al., 1996; Madshus and Stenmark, 1992), for example (Ribosome inactivating proteins) RIPs which enter the cytosol and are among the most potent cytotoxins known. Ribosome-inactivation is achieved in all cases through the cleavage of an N-glycosidic bond between ribose and a specific adenine residue in the universally conserved sequence 5'-AGUACGA*GAGGA-3' (where A* indicates the target adenine) located 250-400nt from the 3' end of

23S/25S/28S rRNAs (Endo and Tsurugi, 1987), (Hartley et al., 1996). Ribosomes depurinated in this manner are unable to bind the EF-2/GTP complex and protein synthesis is blocked at the translocation step (Montanaro et al., 1975). A single RIP molecule is able to depurinate 1000-2000 mammalian cell ribosomes per min under physiological conditions (Eiklid et al., 1980; Endo and Tsurugi, 1988).

- proteins and enzymes such as RNase A that are potent cytotoxins (Leland et al., 1998). These cytotoxic ribonucleases degrade cellular RNA and cause cell death and can be used to kill infected cells and so prevent the proliferation and spread of a pathogen.

These are examples of proteins which will inhibit the replication of a pathogen at a RNA, DNA or protein level by either binding directly to a pathogen protein, replication intermediate or a host factor that is necessary for pathogen replication or movement or transmission and the pathogen life cycle. This strategy is particularly suitable for inactivating viral pathogens. In addition, toxins, such as RIPs or RNase A are described that are suitable for causing cell death on pathogen entry and so halting the spread of infection or proliferation of a pathogen.

In principle all antibodies, proteins, peptides and enzymes that have an activity, that may or may not be enzymatic, which are able to interfere with pathogen life cycles are suitable as part of the present constructs.

In a preferred embodiment of the present invention said enzyme is chitinase or glucanase, glucose oxidase, superoxide dismutase, DNAse or RNAse or RIP or active fragments thereof either singly or in any combination(s).

Constructs

Gene constructs may comprise the following or any combination of the follow and may be encoded on one or more plasmids: Gene constructs may comprise a nucleotide sequence or nucleotide sequences encoding complete recombinant full-size antibodies, dimeric secretory IgA antibodies, multimeric IgM antibodies, the $F(ab')_2$ fragment, the Fab-fragment, the Fv-fragment, single chain antibody fragments (scFvs), single binding domains (dAbs), a bivalent scFv (diabody) (Poljak, 1994), minibody (Carter and Merchant, 1997), bispecific scFv antibodies (Plückthun and Pack, 1997; Fischer et al. *Eur. J. Biochem.* 262, 810-816 (1999)) where the antibody

molecule recognises two different epitopes that may come from the pathogen or the host or both, triabodies and any other part of the antibody (molecular recognition units (MRUs)) which shows binding to the target epitopes. Genes encoding these suitable recombinant antibody fragment(s) may be derived from vertebrates, camelids, avian or pisces species.

In the constructs according to the invention, the antibody is preferably fused to a complete sequence of a toxic agent or a part thereof which still has activity, or which is still functionally active. Also, the chimeric protein may be encoded by nucleotide sequences on one or more constructs and may be assembled *in vivo* by the plant or expression organisms protein assembly and translation machinery. The chimeric protein can also be obtained by biochemical assembly or *in vitro* or *in vivo* assembly of the chimeric immunotoxin subunits using the cells endogenous protein assembly machinery. The antibody, antibodies or fragments thereof are fused directly to the toxic agent or linked by a flexible spacer which does not interfere with the structure or function of the two proteins. Such flexible linkers include copies of the (Glycine-Glycine- Glycine- Glycine-Serine)$_n$ linker, where n is 1 to 4 or more copies of the linker unit, the Genex 212 and 218 linker and the flexible linker peptide of *Trichoderma reesi* cellobiohydrolase I (CBHI) (Turner et al., 1997), (Tang et al., 1996).

Constructs for cellular targeting and membrane localisation

In this invention, this targeting approach has the advantage that the molecular pathogenicide or antibody or fragment thereof can be expressed where the pathogen is most vulnerable to the action of the molecular pathogenicide and/or antibody or fragment thereof.

The desired cellular location of the molecular pathogenicide, or any components thereof, can be achieved by using the appropriate cellular targeting signals, these include but are not limited to signal peptides, targeting sequences, retention signals, membrane anchors, post translational modifications and/or membrane transmembrane domains that target the protein to the desired organelle, desired membrane (plasma membrane, ER, Golgi, nucleus, chloroplast or vacuole) or desired membrane orientation (cytoplasmic or lumenal or plant cell membrane display) (Kim et al., 1997; Rose and Fink, 1987). Localisation sequences can be

targeting sequences which are described, for example in chapter 35 (protein targeting) of L. Stryer *Biochemistry* 4th edition, W.H. Freeman, 1995. Proteins synthesised without a functional signal peptide are not co-translationally inserted into the secretory pathway and remain in the cytosol. Proteins that carry a signal peptide that directs them to the secretory pathway, which may include a transmembrane sequence or membrane anchor, will be targeted for secretion by default or reside in their target membrane organelles. Targeting signals can direct proteins to the ER, retain them in the ER (LYSLYS motif and KDEL), TGN 38, or will target proteins to cell organelles such as the chloroplasts, vacuole, nucleus, nuclear membrane, peroxisomes and mitochondria. Examples for signal sequences and targeting peptides are described in (von Heijne, 1985) (Bennett and Osteryoung, 1991) (Florack et al., 1994). In addition, the targeting signals may be cryptic and encoded by a host plant cell or heterologous eukaryotic cell proteins or animal proteins where the localisation is known and where the protein can be cloned. By constructing a fusion protein with this protein, a molecular pathogenicide can be targeted to the localisation of the protein without the need for identification of the cryptic targeting signal. Suitable cryptic signals are those encoded by the resident Golgi enzymes.

The molecular pathogenicides described in this invention can be targeted to cellular membranes by incorporating heterologous sequences into the recombinant protein which permit its synthesis as a membrane protein or as a membrane associated protein or its post translational modification to associate it with cellular membranes. Suitable membrane anchor sequences, enabling the integration of recombinant antibody fusion proteins and parts thereof in the plasma membrane, include the human T cell receptor transmembrane domains (Gross and Eshhar, 1992), glycophosphatidyl inositol (GPI) anchors (Gerber et al., 1992), immunoglobulin superfamily membrane anchors, tetraspan family members (Tedder and Engel, 1994; Wright and Tomlinson, 1994) and any transmembrane sequence(s) from a known protein or synthesised sequences that have a similar function and can be included in the target protein by recombinant DNA technology.

In addition, the antibodies or parts thereof, or the recombinant antibody fusion proteins, or parts thereof, may be targeted to cell membranes where they could face the cytosolic side of the membrane. Suitable targeting sequences for cytoplasmic display, include the transmembrane domains of: KAR1, for nuclear membrane integration (Rose and Fink, 1987), middle-T antigen (Kim et al., 1997), for plasma

membrane integration and cytochrome b5, for ER membrane integration (Kim et al., 1997). C-terminal linkages to fatty acids using consensus amino acid sequences leading to post translational prenylation, farnesylation, palmitoylation, myristoylation or ankyrin sequence motifs can also be used.

Constructs for antibody stabilisation by membrane display

Pathogen-specific recombinant antibodies can be fused to different transmembrane anchors to improve the expression levels and stability of these molecules inside the plant cell, by targeting the expressed recombinant protein to cell membranes in various orientations. This can be accomplished by adding:

a) C-terminal localisation sequences to target and integrate recombinant cytosolic proteins with N-terminal leader peptides into the bilayer of cellular membranes, thus facing to the plant apoplast. Suitable membrane localisation sequences include the human T cell receptor β chain transmembrane domain and the human platelet derived growth factor receptor (PDGFR) transmembrane domain, glycophosphatidyl inositol (GPI) anchors, immunoglobulin superfamily membrane anchors and any transmembrane sequence(s) from a known protein or synthesised sequences that have a similar function and can be included in the target protein by recombinant DNA technology.

b) Amino terminal transmembrane proteins with either dual or tetrameric plasma membrane spanning domains to expose both the N- and C-termini of secretory recombinant proteins to the cytosol. This can be achieved by using suitable members of the tetraspan family including CD9, CD20, CD81 and the In-Hc-Ic dualspan typeII-IV hybrid of the MHC invariant chain and H-2^d hybrid protein. This method enables the orientation of a secreted and membrane anchored antibody construct with its N- and C-terminus into the cytosol. Alternatively fusions to SNAP-25 can be used for the same orientation.

c) C-terminal anchor sequences to target and integrate recombinant cytosolic proteins without N-terminal leader peptides into the bilayer of endomembranes posttranslationally. Suitable targeting sequences include transmembrane domains of KAR1 for nuclear membrane integration (Rose and Fink, 1987), middle-T antigen for plasma membrane integration (Kim et al., 1997) and cytochrome b5 for ER membrane integration (Kim et al., 1997).

d) Addition of consensus motifs to the protein that permit C-terminal linkages to fatty acids by prenylation, farnesylation, palmitoylation, myristoylation in the cytosol which then lead to membrane integration.

e) Addition of ankyrin sequence motifs (Lambert and Bennett, 1993; Peters and Lux, 1993).

Constructs for *in vivo* protein complex assembly

In addition, the antibody or fragment thereof can be encoded by a separate nucleotide sequence to that of the toxin and the antibody and toxin, either of which may encode membrane localisation or cellular targeting sequences, can be encoded by one or more vectors, e.g., plasmids. The constructs contain nucleotide sequences encoding complimentary binding proteins so that when the antibody, or fragment thereof, is genetically fused to one binding partner and the toxin, or fragment thereof, is genetically fused to the second binding partner, these two independent proteins will bear mutually recognising binding activities. When these two independent proteins are expressed in the same plant compartment, the binding domains will bind to form a molecular pathogenicide with two subunits and similar properties to an antibody-toxin fusion protein. Suitable binding domains/partners include:

- A single chain antibody and its corresponding epitope, where the single chain binds to the epitope and thereby enables binding between two independent proteins,
- leucine zippers (Carter et al., 1995),
- Antibody heavy and light chains, where one protein is fused to the heavy chain and assembly of heavy and light chain takes place in the ER,
- other homo- or hetero-binding domains.

Anyone of ordinary skill in the art will recognise that the component antibody, antibodies or fragments thereof or component pathogen binding peptides, as described, and component toxin or fragments thereof can each bear a binding partner. When expressed in the same compartment of a plant, or when encountering each other, these binding domains can then permit the assembly of a molecular pathogenicide with all the properties required from the components. Anyone skilled in the art will recognise that this can be achieved by other means than those described

above which are intended as examples to better illustrate the principle of *in vivo* assembly and are not intended to be taken as a limiting or a comprehensive description.

Carrier proteins

Anyone skilled in the art will also recognise that the various components of the present invention can be expressed in such a way that they are on the surface of a third carrier protein, suitable carriers include glutathione S-transferase (GST) encoded by *Schistosoma japonicum* (Smith and Johnson, 1988), TMV coat protein, maltose-binding protein and thioredoxin (LaVallie et al., 1993) or other proteins.

In addition, any of the components of the present invention may be tagged with a genetically encoded fluorophore, suitable fluorophores include, but are not limited to, the green fluorescent protein (GFP) from *Aequoria victoria*. This approach would be especially useful for monitoring the localisation of a pathogen or molecular pathogenicide during infection.

If the fusion protein or proteins are expressed in a heterologous organism for production of the protein or proteins, it may be necessary to modify the gene construct in order to match the codon preference of the organism and to remove mRNA motifs that reduce the stability of the transcript.

All of the components of the molecular pathogenicides described in this invention can be separately transformed into plant lines which can then be sexually crossed to give offspring that product the molecular pathogenicides in a functional form.

Anyone skilled in the art will recognise that the antibodies, peptides and toxins can be combined in several forms and encoded on different plasmids to produce proteins that have the desired effect on the pathogen. Anyone skilled in the art will also recognise that assembling the molecular pathogenicides from individually genetically encoded subunits can be achieved by several methods.

Target pathogens

Viruses, bacteria, mycoplasmas, fungi, nematodes, insects and other pathogens.

Vectors

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain a polynucleotide according to the invention or any one of the above-described gene constructs. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Alternatively, the polynucleotides and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

In a preferred embodiment, the polynucleotide present in the vector is linked to regulatory elements which allow the expression of the polynucleotide in prokaryotic and/or eukaryotic cells. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotic and/or eukaryotic cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise normally promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilisation of the transcript, for example, those of the 35S RNA from Cauliflower Mosaic Virus (CaMV). In this respect, the person skilled in the art will readily appreciate that the polynucleotides encoding at least one of the above-described domains of the fusion proteins or pathogenicide of the invention may encode all of the domains or only one. Likewise, said polynucleotides may be under the control of the same promoter or may be separately controlled for expression. Other promoters commonly used are the Figwort Mosaic virus promoter, the polyubiquitin promoter, and the actin promoter for ubiquitous expression. The termination signals usually employed are from the Nopaline Synthase or CaMV 35S gene. A plant translational enhancer often used is the TMV omega sequences, the inclusion of an intron (Intron-1 from the Shrunken gene of maize, for example) has been shown to increase expression levels by up to 100-fold. (Maiti et al., Transgenic Research 6 (1997), 143-156; Ni et al., Plant Journal 7 (1995), 661-676). Additional regulatory elements may include transcriptional as well as translational enhancers.

Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the P_L, *lac*, *trp* or *tac* promoter in *E. coli*, and examples for regulatory elements permitting expression in eukaryotic host cells are the *AOX1* or *GAL1* promoter in yeast or the CMV-, SV40- , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pSPORT1 (GIBCO BRL). Advantageously, the above-described vectors of the invention comprises a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed hosts, for example plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and hygro, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilise indole in place of tryptophan; hisD, which allows cells to utilise histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilise mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from Aspergillus terreus which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338).

Useful scorable markers are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or ß-glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and organisms containing a vector of the invention.

Host cells and expression of fusion proteins and pathogenicides

The present invention furthermore relates to host cells comprising a vector as described above or a polynucleotide according to the invention. The vector or polynucleotide according to the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extrachromosomally.

The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred fungal cells are, for example, those of the genus Saccharomyces, in particular those of the species S. cerevisiae.

Another subject of the invention is a method for the preparation of the above-described fusion proteins and pathogenicides which comprises the cultivation of host cells according to the invention which, due to the presence of a vector or a polynucleotide according to the invention, are able to express such a protein, under conditions which allow expression and optionally assembly of the fusion protein or pathogenicide and recovering of the so-produced protein from the culture. Depending on the specific constructs and conditions used, the protein may be recovered from the cells, from the culture medium or from both. For the person skilled in the art it is well known that it is not only possible to express a native protein but also to express the protein as fusion polypeptides or to add signal sequences directing the protein to specific compartments of the host cell, e.g., ensuring secretion of the peptide into the culture medium, etc. Furthermore, such a protein and fragments thereof can be chemically synthesised and/or modified according to standard methods described, for example herein.

The present invention furthermore relates to molecular pathogenicides encoded by the polynucleotides according to the invention or produced by the above-described method. In this context, it is also understood that the fusion proteins and pathogenicides according to the invention may be further modified by conventional methods known in the art.

Plant promoters and expression control elements

The fusion constructs are expressed in plants either stably in transgenic plants or transiently under the control of any type of promoter that is active in plants. For long-term resistance in host plants, high yield production of recombinant proteins, stable expression is preferred.

In general, such regulatory elements comprise a promoter active in plant cells. To obtain expression in all tissues of a transgenic plant, preferably constitutive promoters are used, such as the 35 S promoter of CaMV (Odell, Nature 313 (1985), 810-812) or promoters of the polyubiquitin genes of maize (Christensen, Plant Mol. Biol. 18 (1982), 675-689). In order to achieve expression in specific tissues of a transgenic plant it is possible to use tissue specific promoters (see, e.g., Stockhaus, EMBO J. 8 (1989), 2245-2251). Further examples are:

a) Expression control elements (*e.g.* promoters listed below in b to f, enhancer sequences, transcriptional and translational enhancers, transcription terminators, polyadenylation sites etc.) and a selectable marker if necessary.
b) Constitutive promoters such as the CaMV-35S (Benfey et al., 1989) and the *nos* promoter (Mitra and Gynheung, 1989).
c) Viral subgenomic promoters.
d) Tissue specific promoters and chimeric promoters (Ni et al., 1995), (Comai et al., 1990).
e) Inducible promoters (Caddick et al., 1998).
f) Transient expression systems (Kapila et al., 1997).

Known are also promoters which are specifically active in tubers of potatoes or in seeds of different plants species, such as maize, Vicia, wheat, barley etc. Inducible promoters may be used in order to be able to exactly control expression. An example for inducible promoters are the promoters of genes encoding heat shock proteins. Also microspore-specific regulatory elements and their uses have been described (WO96/16182). Furthermore, the chemically inducible Tet-system may be employed (Gatz, Mol. Gen. Genet. 227 (1991); 229-237). Further suitable promoters are known to the person skilled in the art and are described, e.g., in Ward (Plant Mol. Biol. 22 (1993), 361-366). The regulatory elements may further comprise transcriptional and/or translational enhancers functional in plants cells. Furthermore, the regulatory elements may include transcription termination signals, such as a poly-A signal, which lead to the addition of a

poly A tail to the transcript which may improve its stability.

Furthermore, it is in principle possible to modify the coding sequence in such a way that the protein is located in any desired compartment of the plant cell. These include the endoplasmatic reticulum, the vacuole, the mitochondria, the plastids, the apoplast, the cytoplasm etc. Methods how to carry out this modifications and signal sequences ensuring localisation in a desired compartment are well known to the person skilled in the art.

Transformation

Methods for the introduction of foreign DNA into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, biolistic methods like particle bombardment and other methods known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of Agrobacterium which allow for stably integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example cotransformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilise enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Suitable strains of Agrobacterium tumefaciens and vectors as well as transformation of Agrobacteria and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad.

Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialised vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of Agrobacterium tumefaciens is preferred in the method of the invention, other Agrobacterium strains, such as Agrobacterium rhizogenes, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, Plant Physiol. 104 (1994), 37-48; Vasil, Bio/Technology 11 (1993), 1553-1558 and Christou (1996) Trends in Plant Science 1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), Gene Transfer To Plants. Springer Verlag, Berlin, NY (1995).

The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc.

Transformation can be done using any method that leads to expression of construct or constructs in a plant and these methods can be used for stable transformation where the gene of interest is incorporated in the host plant DNA or where the construct is transiently expressed. Examples of transformation technology include:

a) *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* mediated transformation (Turpen et al., 1993; White, 1992): based on the insertion of a foreign DNA sequence into the plant genome carried on a plasmid DNA within the agrobacteria. The foreign gene is inserted into the plant genome together with bacterial plasmid sequences.

b) Particle bombardment (Sanford et al., 1990), (Klein and Fitzpatrick-McElligott, 1993) or biolistic process (Furth, 1997): Particle bombardment uses particles coated with the DNA that penetrate the plant cell at high velocity and the DNA is incorporated into the host genome by host recombination processes. Besides

particle bombardment biolistic processes also include injection methods.

c) Tissue electroporation (Chowrira et al., 1995; D'Halluin et al., 1992): under the influence of an electric field, DNA enters pores in the plant cell membrane and is incorporated into the plant genome by recombination.
d) Use of liposomes or methods which increase the uptake of free DNA (Spörlein and Koop, 1991; White, 1992).
e) Any method for integration of foreign DNA in a plant cell resulting in transiently or stably transformed plants.

Target plants

The present invention relates to transgenic plant cells which contain a polynucleotide, vector or composition of vectors of the invention. Preferably, said polynucleotide or vector is stably integrated into the genome.

As is immediately evident to the person skilled in the art, the vectors of the present invention can carry nucleic acid molecules encoding the domains of the antibody, fusion protein or pathogenicide of the invention either alone or in combination. The same applies to the above described plant cells, plant tissue and plants transformed therewith. Likewise, said nucleic acid molecules may be under the control of the same regulatory elements or may be separately controlled for expression. In this respect, the person skilled in the art will readily appreciate that the nucleic acid molecules encoding the domains of the fusion protein or pathogenicide can be expressed in the form of a single mRNA as transcriptional and optionally translational fusions. This means that domains are produced as separate polypeptides or in the latter option as a fusion polypeptide that is further processed into the individual proteins, for example via a cleavage site for proteinases that has been incorporated between the amino acid sequences of both proteins. The resultant protein domains can then self-assemble in vivo. Of course, the domains may also be expressed as a bi- or multifunctional polypeptide, preferably disposed by a peptide linker which advantageously allows for sufficient flexibility of both proteins. Preferably said peptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of one of said proteins and the N-terminal end of the other of said proteins when said polypeptide assumes a conformation suitable for biological activity of both proteins when disposed in aqueous solution in the plant cell. Examples

of the above-described expression strategies can be found in the literature, e.g., for dicistronic mRNA (Reinitiation) in Hefferon, J. Gen. Virol. 78 (1997), 3051-3059, fusion proteins are described in Brinck-Peterson, Plant Mol. Biol. 32 (1996), 611-620 and Hotze, FEBS Lett. 374 (1995), 345-350; bifunctional proteins are discussed in Lamp, Biochem. Biophys. Res. Com. 244 (1998), 110-114 and Dumas, FEBS Lett. 408 (1997), 156-160 and for linker peptide and protease it is referred to Doskeland, Biochem. J. 313 (1996), 409-414.

In a preferred embodiment of the invention, the transgenic plant cell comprises a selectable marker. As described above, various selectable markers can be employed in accordance with the present invention. Advantageously, selectable markers may be used that are suitable for direct selection of transformed plants, for example, the phophinothricin-N-acetyltransferase gene the gene product of which detoxifies the herbicide L-phosphinothricin (glufosinate or BASTA); see, e.g., De Block, EMBO J. 6 (1987), 2513-2518 and Dröge, Planta 187 (1992), 142-151.

The presence and expression of the polynucleotides or vectors in the transgenic plant cells leads to the synthesis of a fusion protein, antibody or pathogenicide of the invention or assembly of the same which has an influence on pathogen resistance in plants containing such cells.

Thus, the present invention also relates to transgenic plants and plant tissue comprising transgenic plant cells according to the invention. Due to the expression of a fusion protein, the antibody against the viral movement and/or replicase protein or pathogenicide of the invention or their domains, e.g., in cellular compartments and/or plant tissue these transgenic plants may show various physiological, developmental and/or morphological modifications in comparison to wild-type plants. Advantageously, these transgenic plants display a resistance against a pathogen that the corresponding wild type plant was susceptible to.

In general, the plants which can be modified according to the invention can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture, such as crop plants (e.g. maize, rice, barley, wheat, rye,

oats etc.), potatoes, oil producing plants (e.g. oilseed rape, sunflower, pea nut, soy bean, etc.), cotton, sugar beet, sugar cane, leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

In yet another aspect, the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention. Harvestable parts can be in principle any useful parts of a plant, for example, leaves, stems, fruit, flowers, seeds, roots etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

Kits

In addition, the present invention relates to a kit comprising the above-described antibodies, fusion protein, pathogenicide, polynucleotide or vectors. The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic plant cells, plant tissue or plants. The kit of the invention may advantageously be used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art. The kit or its ingredients according to the invention can be used in plant cell and plant tissue culture, for example in agriculture. The kit of the invention and its ingredients are expected to be very useful in breeding new varieties of, for example, plants which display improved properties such as those described herein.

It is also immediately evident to the person skilled in the art that the polynucleotides and vectors of the present invention can be employed to produce transgenic plants with a further desired trait due to genetic engineering (see for review TIPTEC Plant Product & Crop Biotechnology 13 (1995), 312-397). This can be, for example, an acquired resistance to other pathogens or quality improvements of the plants comprising (i) herbicide tolerance (DE-A-3701623; Stalker, Science 242 (1988), 419), (ii) insect resistance (Vaek, Plant Cell 5 (1987), 159-169), (iii) virus resistance (Powell, Science 232 (1986), 738-743; Pappu, World Journal of Microbiology &

Biotechnology 11 (1995), 426-437; Lawson, Phytopathology 86 (1996), 56 suppl.), (vi) ozone resistance (Van Camp, BioTech. 12 (1994), 165-168), (v) improving the preserving of fruits (Oeller, Science 254 (1991), 437-439), (vi) improvement of starch composition and/or production (Stark, Science 242 (1992), 419; Visser, Mol. Gen. Genet. 225 (1991), 289-296), (vii) altering lipid composition (Voelker, Science 257 (1992), 72-74), (viii) production of (bio)polymers (Poirer, Science 256 (1992), 520-523), (ix) alteration of the flower colour, e.g. by manipulating the anthocyanin and flavonoid biosynthetic pathway (Meyer, Nature 330 (1987), 667-678, WO90/12084), (x) resistance to bacteria, insects and fungi (Duering, Molecular Breeding 2 (1996), 297-305; Strittmatter, Bio/Technology 13 (1995), 1085-1089; Estruch, Nature Biotechnology 15 (1997), 137-141), (xi) inducing and maintaining male and/or female sterility (EP-A1 0 412 006; EP-A1 0 223 399; WO93/25695) and (xii) remediation of contaminated soils (Cunningham, TIBTECH 13 (1995), 393-397).

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilised which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

Description of the figures

Figure 1 shows a description of various orientations for molecular pathogenicide display on cellular membranes. Recombinant molecular pathogenicides can be targeted by cellular signals and expressed in several orientations on cellular membranes, for example: A: where the recombinant protein faces the cytosol or

extracellular space after fusion to a transmembrane domain or after post translational lipid modification and B: where the recombinant protein is fused to a protein with 4 transmembrane domains. In C and D possible orientations of toxins are displayed.
In addition, the toxin and or recombinant antibody fragment can be fused to the c terminal of any of the example protein structures.
N: protein amino terminal; C: protein carboxy terminal; tm: transmembrane domain; rAb: recombinant antibody fragment or binding domain.

Figure 2 shows example constructs for membrane anchoring of scFv24 in the plant cell plasma membrane (see example 1). 35SS: 35S promoter from Cauliflower Mosaic Virus with duplicated enhancer; CHS 5'-UT: chalcone synthase 5' untranslated region; Leader peptide: original murine leader sequence from the parental monoclonal antibody 24 light chain; V_L: Variable domain of the parental monoclonal antibody 24 light chain; V_H: Variable domain of the parental monoclonal antibody 24 heavy chain; Linker: 14 amino acid linker sequence; c-myc: c-myc epitope tag sequence; TcRβ: Human T cell receptor β chain; PDGFRTM: Platelet derived growth factor receptor transmembrane domain; Term: termination sequence from Cauliflower mosaic virus.

Figure 3 shows example constructs for molecular pathogenicide display facing the cell cytoplasm. 35SS: 35S promoter from Cauliflower Mosaic Virus with duplicated enhancer; CHS 5'-UT: chalcone synthase 5' untranslated region; VL: Variable domain of the parental monoclonal antibody 24 light chain; VH: Variable domain of the parental monoclonal antibody 24 heavy chain; Linker 1: 14 amino acid linker (Genex 212) sequence; Linker 2: 10 amino acid linker (Gly4Ser)2 sequence; Term: termination sequence from Cauliflower mosaic virus.

Figure 4 shows example constructs for viral coat protein antibody fusion proteins and various potential carrier antibody-protein fusion proteins. scFv24: single chain antibody derived from parental monoclonal mAb24 recognising a neotope on the surface of intact TMV virions; GST: Glutathione S-transferase from *Schistosoma japonicum*; Omega sequence: Tobacco Mosaic virus 5' untranslated region; linker: 10 Amino acid $(Gly_4Ser)_2$ linker sequence; His6: 6 histidine residue epitope tag sequence; 35SS: 35S promoter from Cauliflower Mosaic Virus with duplicated

enhancer; TRXec: Thioredoxin from *Escherichia coli*; TRXnt: Thioredoxin from *Nicotiana tabacum*; CP: coat protein monomer from Tobacco mosaic virus; TMV 3' UT: Tobacco Mosaic virus 3' untranslated region.

Figure 5 shows the strategy and example constructs for *in vivo* molecular pathogenicide assembly using an antibody: antigen interaction as the binding partners for *in vivo* assembly. The two binding partners are an epitope tag and a high affinity antibody which specifically recognises this epitope tag. To assemble a molecular pathogenicide protein complex, the epitope specific antibody is genetically fused to a pathogen specific antibody and the epitope tag is genetically fused to the toxin sequence. Both of these recombinant proteins are then expressed in the same cell compartment. The epitope specific antibody binds the epitope expressed on the surface of the toxin. This high affinity interaction then gives a molecular pathogenicide protein complex, which specifically recognises the pathogen and bears a toxic activity. Linker 4 can encode specific protease cleavage sites.
The epitope and pathogen specific antibodies can also be included in the constructs in the same orientation but where the epitope specific antibody precedes the pathogen specific antibody in the 5' to 3' direction.

A: schematic of molecular pathogenicide protein complex assembly in a cell compartment; B: Example constructs showing two possible arrangements (Ab1 and Ab2) of the individual V_L and V_H domains of both the pathogen specific and epitope specific antibody fragments; C: two possible arrangements (Tox 1 and Tox2) for epitope toxin fusion proteins.

Figure 6 shows the strategy and example constructs for *in vivo* molecular pathogenicide assembly using an antibody heavy chain: antibody light chain interaction as the binding partners for *in vivo* assembly. The two binding partners are an antibody heavy chain and an antibody light chain which specifically recognises this epitope tag. To assemble a molecular pathogenicide protein complex, the epitope specific antibody is genetically fused to a pathogen specific antibody heavy chain C-terminus. Both of these recombinant antibody heavy chain and light chains are then expressed in the same cell compartment, where they assemble via disulphide bridges to give a molecular pathogenicide protein complex, which

specifically recognises the pathogen and bears a toxic activity. Linker 1 can encode specific protease cleavage sites or the epitope specific antibody that recognizes an epitope tagged toxin. Also, the toxin can fused to the N-terminus of the antibody heavy chain using linker 1, or the N or C terminus of the light chain.
A: schematic of the final assembled molecular pathogenicide. B: example constructs.

Figure 7 shows a cDNA construct for targeting and expression of scFv24 on plant cell membranes. cDNAs of mAb24 variable light (V_L) and heavy chain (V_H) domains connected by a 14 amino acid linker were fused to the the human TcRβ transmembrane domain and cloned into *EcoR*I and *Xba*I restriction sites of the plant expression vector pSS (33). The DNA sequence of the *EcoR*I/*Xba*I fragment from pscFv24-TcRβ is depicted in SEQ ID NO: 3. 35SS = double enhanced CaMV-35S promoter; CHS-5'-UT = 5' untranslated region of the chalcone synthase; LP = signal sequence of the murine mAb24 light chain; c-*myc* = c-*myc*-epitope tag; TM = transmembrane domain; TCaMV = CaMV termination sequence.

Figure 8 shows the levels of functional scFv24-TcRβ in transgenic *N. tabacum* cv. BY-2 suspension cell lines. scFv24 production levels in tobacco BY-2 cell extracts and the culture supernatant were analyzed by ELISA using the anti-mAb24 antisera and are indicated as ng scFv24 per g cell material. $T1_{BY\text{-}2}$-$T2_{BY\text{-}2}$ = transgenic BY-2 supension cell lines producing scFv24-TcRβ.

Figure 9 shows Western blot analysis of a T_1 tobacco plant producing scFv24-TcRβ. Equivalent amounts of protein from intercellular washing fluids and total soluble proteins from one T_1 plant producing scFv24-TcRβ (lane 1 and 2) were separated on a 12 % (w/v) reducing SDS-PAGE gel and transferred to nitrocellulose. Recombinant protein was detected by using a rabbit anti-mAb24 antisera as primary antibody and goat-anti rabbit antibody conjugated to alkaline phosphatase as a secondary antibody and followed by NBT/BCIP staining. Estimated molecular weights of recombinant proteins are indicated (Marker). IWF = intercellular washing fluid; TSP = total soluble protein.

Figure 10 shows the subcellular localization of membrane anchored scFv24-TcRβ in transgenic *N. tabacum* cv. BY-2 protoplasts by indirect immunofluorescence. Fixed

protoplasts from non-transgenic BY-2 cells (A) and line $T2_{BY-2}$ producing scFv24-TcRβ (B, C, D) were labeled either using anti-mAb24 antisera as a primary antibody followed by a FITC conjugated goat-anti rabbit secondary antibody (A, B, D) or using anti-human TcRβ antibody as a primary antibody, followed by an FITC conjugated goat-anti mouse secondary antibody (C). Magnification = x 400.

Figure 11 shows immunogold localization of scFv24- in transgenic *N. tabacum* cv. Petite Havana SR1 leaves. Ultrathin-sections from plant line $T4_{SR1}$ producing scFv24-TcRβ were probed with rabbit anti-mAb24 antisera primary antibody and 12 nm gold particle conjugated goat-anti rabbit secondary antibody. Arrowheads indicate immunogold labelling of membrane anchored scFv24-TcRβ plant cell plasma and nuclear membrane.

Figure 12 shows cDNA constructs for targeting and expression of cytosolic scFv24 to plant cell membranes. scFv24 cDNA, comprising variable light chain (V_L) and heavy chain (V_H) domains connected by a 14 amino acid 212 linker (linker 2), were fused to the C-terminal transmembrane domains using the Gly_4Ser linker (linker 1) and subcloned into the plant expression vector pSS. A) Cytoplasmic targeting vectors containing a C-terminal transmembrane domain. B) Cytoplasmic targeting control vector lacking a transmembrane domain (construction of this vector is described in: Zimmermann et al., 1998). 35SS = enhanced CaMV-35S promoter; CHS 5'UT = 5' untranslated region of chalcone synthase; KAR1 = transmembrane domain of KAR1 for nuclear membrane integration; mT = transmembrane domain of middleT antigen plasma membrane integration; cytb5 = transmembrane domain of cytochrome b5 for ER membrane integration; syn1 = transmembrane domain of synaprobrevin1 for membrane integration; His6 = histidine 6-tag; c-*myc* = c-*myc*-epitope tag; TCaMV = CaMV termination sequence. The DNA sequence of the *EcoR*I/*Xba*I fragment from pscFv24-kar1 is depicted in SEQ ID NO: 12. Codon usage of the yeast KAR1 transmembrane domain was adapted to tobacco, wheat and pea; except the TGT (Cys) and CTT (Leu) codons which are rare codons for wheat. The Ile codon ATA (in the transmembrane domain) is also a rare codon for wheat and the Leu codon CTG (in the transmembrane domain) is a rare codon for pea and tobacco, but both codons were necessary to introduce a *Bst*1107 restriction site. The DNA sequence of the *EcoR*I/*Xba*I fragment from pscFv24-mT is shown in SEQ ID NO: 13. Codon usage of

the transmembrane domain of murine polymovirus middle-T antigen was adapted to tobacco, wheat and pea. The Ala codon GCG and the Leu codon CTG (both in the transmembrane domain) are rare codons for tobacco and pea, but both were neccessary to introduce a *Eco*47-3 restriction site. The DNA sequence of the *EcoR*I/*Xba*I fragment from pscFv24-cytb5 is shown in SEQ ID NO: 14. Codon usage of rat liver cytochrome b5 transmembrane domain was adapted to tobacco, wheat and pea; except the very C-terminal codon (Asp) which is a rare codon for wheat. The Ala codon GCG (in the transmembrane domain) is also a rare codon for pea and tobacco and the Ile codon ATA (in the transmembrane domain) is a rare codon for wheat, but both codons are necessary to introduce a *Eco*RV retriction site. The DNA sequence of the *EcoR*I/*Xba*I fragment from pscFv24-syn1 is depicted in SEQ ID NO: 15. Codon usage of human synaptobrevin transmembrane domain was adapted to tobacco, wheat and pea; except the last codon (Asp) which is a rare codon for wheat.

Figure 13 shows a cDNA construct for targeting and expression of scFv24-PE400. scFv24 cDNA, comprising variable light chain (V_L) and heavy chain (V_H) domains connected by a 14 amino acid 212 linker (linker 2), were fused to the C-terminal *Pseudomonas* exotoxin domain III using a cellobiohydrolase I (CBH) linker of *Trichoderma reesi* and subcloned into the plant expression vector pSS. 35SS = enhanced CaMV-35S promoter; CHS 5'UT = 5' untranslated region of chalcone synthase; LP = signal sequence of the murine mAb24 light chain; linker 1 = Gly_4Ser linker; tag54 = epitope tag; TCaMV = CaMV termination sequence. The DNA sequence of the *EcoR*I/*Xba*I fragment from pscFv24-PE400 is shown in SEQ ID NO: 18.

Figure 14: Molecular pathogenicide based on a single chain (scFv24) fusion to *E. coli* RNAseE. The two domains of the pathogenicide were connected by a short Gly-Gly-Gly-Ser linker peptide. This set up can be modified in multiple ways by using different scFv antibodies binding to structural and nonstructural viral target proteins, other RNAse genes or domains thereof fused to either the N- or the C-terminus of any selected scFv cDNA.

Figure 15 shows constructs for expression of scFv24 fusion proteins in the cytoplasm and ER of plant cells. scFv24 cDNA, comprising variable light chain (V_L)

and heavy chain (V_H) domains connected by a 14 amino acid 212 linker (linker 2), were fused to CP using the $(Gly_4Ser)_2$ linker (linker 1) and subcloned into the plant expression vector pSS. A) Cytoplasmic targeting vectors containing a C-terminal His6 or KDEL sequence. B) Cytoplasmic targeting control vectors lacking a fusion partner. C) ER retention vector. D) Apoplastic targeting vector (the fusion protein is secreted to the apoplast and will enter the cell by binding to invading TMV virions). 35SS = enhanced CaMV-35S promoter; • = 5' untranslated region of TMV; LP = codon optimized original mouse leader peptide sequence from mAb24; CP = fusion partner TMV coat protein; His6 = histidine 6-tag; KDEL = ER retention signal; Pw = TMV 3' untranslated region. The DNA sequence of the *EcoR*I/*Xba*I fragment from CP-scFv24H is shown in SEQ ID NO: 24. The DNA sequence of the *EcoR*I/*Xba*I fragment from CP-scFv24K is shown in SEQ ID NO: 25. The DNA sequence of the *EcoR*I/*Xba*I fragment from scFv24H is shown in SEQ ID NO: 26. The DNA sequence of the *EcoR*I/*Xba*I fragment from scFv24K is shown in SEQ ID NO: 27. The DNA sequence of the *EcoR*I/*Xba*I fragment from L-CP-scFv24K is shown in SEQ ID NO: 28.

Figure 16 shows protein levels of ER retained scFv- fusion proteins. *N. tabacum* cv. Petite Havana SR1 leaves were transiently transformed with recombinant agrobacteria containing the construct L-CP-scFv24K and incubated for three days. Total soluble protein was isolated and levels of functional scFv24-fusion protein, was quantified in a TMV-specific ELISA and indicated as ng per gram leaf material. The column represents the mean value of four leaves. Standard deviations are indicated.

Figure 17 shows Western blot analysis of ER retained fusion proteins. Affinity purified L-CP-scFv24K was separated by 12% SDS-PAGE and proteins were transferred to a nitrocellulose membrane. Blots were probed with CP-specific mAb29 primary antibody followed by alkaline phosphatase conjugated goat-anti rabbit and goat-anti mouse secondary antibody and NBT/BCIP staining. Lane 1: Prestained protein marker; lane 2: TMV-affinity purified L-CP-scFv24K.

Figure 18 shows levels of cytoplasmic expressed fusion proteins. *N. benthamiana* leaves were transiently transformed with recombinant agrobacteria and incubated for three days. Total soluble protein was isolated and levels of functional scFv24,

expressed as part of the fusion proteins, were quantitated in a TMV-specific ELISA and indicated as ng per gram leaf material. Each column represents the mean value of four leaves and demonstrates the protein levels of constructs CP-scFv24H and scFv24H containing a C-terminal His6 sequence and protein levels of constructs CP-scFv24K and scFv24K containing a C-terminal KDEL sequence. Standard deviations are indicated with bars.

Figure 19 shows the cloning of TMV 30K movement protein specific antibodies into the plant expression vector pSSH1. Single chain fragments obtained by phage display were subcloned into the BISCA2429 expression cassette (Fischer et al., (1999) *Eur. J. Biochem.* 262, 810-816) using *Nco* I/ *Sal* I restriction sites. Resulting temporary constructs (5´- UTR of TMV omega sequence, scFv, His6 tag) were subcloned into the plant expression vector pSSH1 using *Eco* RI/ *Xba* I subcloning assembling the final vector for plant transformation and expression (35 SS promoter, 5´- UTR of TMV omega sequence, scFv, His6 tag, 3´- UTR and Terminator of CaMV) of the anti 30K scFv.

Figure 20 shows the reactivity in ELISA of 10 different 30K-specific scFv antibodies against GST-30K (biotinylated and non biotinylated) and GST (A) and Reactivity in ELISA of the same 10 scFv fragments against five different Domains of the 30K movement protein expressed as GST-fusion proteins (B). The 30K-specific scFvs were selected from a phage library derived from GST-30K immunized mice.

Figure 21 shows the amino acid residues of two selected scFv binding to the 30K movement protein of TMV obtained by phage display using GST-30K immunized mice for PCR-based amplification of V_H- and V_L-fragments. scFv 30-1= 30K specific scFv No. 1, scFv 30-2= 30K specific scFv No. 2. Aminoacid residues were derived from cDNA-sequencing of the respective phage derived scFv-cDNA clones as described (Figure 19).

Figure 22 shows the time course (44h, 48h, 52h, 56h, 62h and 72h post inoculation, p.i.) for monitoring the accumulation of coat protein in transgenic and non-transgenic anti-30K scFv expressing plants detected by western blotting using a TMV coat protein specific antibody (mAb 24) upon inoculation with TMV *vulgare*. SR1= SR1

wild-type plants, NC= negative control (Transgenic SR1 expressing the antitumor scFv T84.66).

Figure 23 shows aminoacid sequences derived from the cDNA sequence of antiviral scFv-antibodies obtained by hybridoma rescue (Figure 24) directed against the 3a movement protein of CMV (23a), a component of the TMV replicase (23b, 54K of TMV) and a plant virus minimal protein (23c, 3min of PLRV).

Figure 24 shows binding competition between antibodies and PLRV ssRNA. 100 ng of the GST-3min protein were incubated for 30 min at RT with either indicated amounts of polyclonal anti-3min antibodies (pAb, lanes 3, 4, 5 and 6), anti-3min antibody (mAb, lanes 7, 8, 9 and 10) and polyclonal anti-GST antibodies (lane 1) or without antibody (lane 2). The complexes were mixed with 0.5 ng of radioactively labeled ssRNA probe, incubated for 25 min at 4°C and irradiated with UV light at RT. After digestion of unprotected RNA by RNase A, the complexes were analysed by 15% SDS-PAGE and the dried gel was autoradiographed.

Figure 25: Epitope mapping of three different antiviral antibodies namely (a) mAb 29 (see example 1), (b) scFv 54-1 (Example 6, Figure 23b, Figure 29) and (c) scFv 3a-2 (Example 6, Figure 23a). Sequences were obtained from phage ELISA positive clones after the third round of biopanning using two peptide display libraries (Cortese et al., (1995) Curr. Opin. Biotechnol. 6, 73-80). Resulting sequences were aligned and the consensus epitope was determined. In each case the epitope could be mapped within the parental sequence of the different viral sequences analysed.

Figure 25a: Epitope mapping and consensus-sequence of mAb 29 using peptide display and two different peptide display libraries (9mer random library (pVIII 9aa) and 9mer (9aa.Cys) constrained library). From the 9mer random library 10 different positive clones could be characterized and sequenced, from the 9mer constrained library 5 positive clones were characterized. Both libraries resulted in the identification of the same epitope based on a consensus sequence which could be mapped within the TMV-coatprotein sequence.

Figure 25b: Epitope mapping and consensus-sequence of scFv 54-1 using peptid display (9mer linear library). From the 9mer linear library 4 different positive clones

could be characterized and sequenced, resulting in the identification of the same scFv epitope based on a consensus sequence which could be mapped within the 54K protein sequence.

Figure 25c: Epitope mapping and consensus-sequence of scFv 3min using peptide display and two different peptide display libraries (9mer random library (pVIII 9aa) and 9mer (9aa.Cys). From the 9mer random library 8 different positive clones could be characterized and sequenced, from the 9mer constrained library 7 positive clones were characterized. Both libraries resulted in the identification of the same epitope based on a consensus sequence which could be mapped within the border region of GST-3min.

Figure 26 shows a cDNA construct for studying assembly of recombinant proteins. Constructs for expression of biscFv2429, PE280-tag29 and PE400-tag29 in the apoplast of plant cells and GST-tag29 in bacteria.

A) scFv cDNAs, composed of mAb24 and mAb29, variable light chain (V_L) and heavy chain (V_H) domains connected by a 14 amino acid 212 linker (linker 2), were fused using the CBHI-linker. biscFv2429 was subcloned into the plant expression vector pSS. B) PE280 composed of PE domain II (aa 280-364) and domain III (aa 381-609) was fused to the epitag-29 via a Gly_4Ser linker (linker 1). PE280-tag29 was subcloned into the plant expression vector pSS. C) PE400 composed of PE domain III (aa 381-609) was fused to the epitag-29 via a Gly_4Ser linker (linker 1). PE400-tag29 was subcloned into the plant expression vector pSS. D) The epitag-29 was fused to the C-terminus of GST via a Gly_4Ser linker (linker 1) in the pGEX-5X-3 vector. The DNA sequence of the *EcoR*I/*Xba*I fragment from PE280-tag29 is shown in SEQ ID NO: 161. The DNA sequence of the *EcoR*I/*Xba*I fragment from PE400-tag29 is shown in SEQ ID NO: 162.

35SS = double enhanced CaMV-35S promoter; Ptac = *tac* promoter; CHS-5'-UT = 5' untranslated region of the chalcone synthase; LP = leader peptide of the murine monoclonal antibody mAb24 light chain; GST = glutathione S-tranferase; his6 = histidine6 tag; tag29 = epitag-29; TCaMV = CaMV termination sequence.

Figure 27 shows the detection of GST-tag29 by immunoblot. Serial dilutions of bacterial produced and affinity purified GST-tag29 in PBS (A) or protein extract of *N. tabacum* cv. Petite Havana SR1 (B) were separated on a 12 % (w/v) reducing SDS-

PAA gel and transferred to nitrocellulose. Recombinant protein was detected by using rAb29 and goat-anti mouse antibody conjugated to alkaline phosphatase as a secondary antibody and followed by NBT/BCIP staining. Amounts of GST-tag29 loaded and molecular weights of the prestained protein marker are indicated.

Figure 28 shows simulation of *in vivo* assembly by ELISA. Assembly of biscFv2429 from transgenic plants and GST-tag29 produced in bacteria followed by affinity purification was analysed by ELISA. The components were added in the following order to the microtiter plate: Polyclonal TMV rabbit-anti TMV antibodies (7μg/ml), 1% BSA for blocking, TMV virions (1μg/ml), plant extracts from a transgenic plant producing biscFv2429 (diluted 1:10 or 1:100), bacterially expressed GST-tag29, mouse anti-GST mAb (1μg/ml), 1:5000 diluted alkaline phosphatase labelled goat anti-mouse Fc antibody and substrate. Controls were performed using extracts from a non-transgenic tobacco plant. The levels of *in vivo* assembly are indicated as OD 405nm. Each column represents the mean value of two independent ELISA experiments. Standard deviations are indicated with bars.

Figure 29 shows immunoblot of PE280-tag29 transient transformed tobacco leaves. Tobacco leaves transformed using recombinant agrobacteria were incubated for 72h and protein extract was isolated. 15μl and 5μl of total soluble proteins from tobacco leaves producing PE280-tag29, 10μl of a non-transgenic plant (WT) and 400ng affinity purified GST-tag29 as control were separated on a 12 % (w/v) reducing SDS-PAGE gel and transferred to nitrocellulose. Recombinant protein was detected by using a mouse anti-GST mAb as primary antibody and goat-anti mouse antibody conjugated to horseradish peroxidase as a secondary antibody, followed by chemiluminescence detection. The arrow indicates the position of the degraded PE280-tag29 band. Molecular weights of a prestained marker are indicated.

Figure 30 shows a cDNA construct for targeting and expression of scFv24 on plant cell membranes. cDNAs of mAb24 variable light (V_L) and heavy chain (V_H) domains connected by a 14 Genex-212 amino acid linker (36) were fused to the PDGFR and cloned into *EcoR*I and *Xba*I restriction sites of the plant expression vector pSS (33). 35SS = double enhanced CaMV-35S promoter; CHS-5'-UT = 5' untranslated region of the chalcone synthase; c-*myc* = c-*myc*-epitope tag; TM = transmembrane domain;

TCaMV = CaMV termination sequence. The DNA sequence of the *EcoR*I/*Xba*I fragment from pscFv24-PDGFR is depicted in SEQ ID NO: 163.

Figure 31 shows the levels of functional scFv24-PDGFR in transgenic *N. tabacum* cv. BY-2 suspension cell lines. scFv24 production levels in tobacco BY-2 cell extracts and the culture supernatant were analyzed by ELISA using the anti-mAb24 antisera and are indicated as ng scFv24 per g cell material. $P1_{BY\text{-}2}$-$P8_{BY\text{-}2}$ = transgenic BY-2 suspension cell lines producing scFv24-PDGFR.

Figure 32 shows Western blot analysis of T_1 tobacco plants producing scFv24-PDGFR. Equivalent amounts of protein from intercellular washing fluids and total soluble proteins from three T_1 plants producing scFv24-PDGFR were separated on a 12 % (w/v) reducing SDS-PAGE gel and transferred to nitrocellulose. Recombinant protein was detected by using a rabbit anti-mAb24 antisera as primary antibody and goat-anti rabbit antibody conjugated to alkaline phosphatase as a secondary antibody (A) or using a murine anti-c-*myc* antibody as primary antibody and goat-anti mouse antibody conjugated to alkaline phosphatase as secondary antibody (B) and followed by NBT/BCIP staining. Estimated molecular weights of recombinant proteins are indicated (marker). IWF = intercellular washing fluid; TSP = total soluble protein.

Examples

The following examples are given to better describe the practice and applications of the present invention and should not be considered to be a limiting description nor interpreted to limit the scope and applications of the present invention. Those skilled in the art will recognise that various modifications can be made to the methods and genes described here without substantively departing from the spirit and scope of the present invention.

Example 1: Expression of a membrane integrated anti-viral antibody

Plasma membrane targeted expression of a recombinant antibody against the coat protein of Tobacco Mosaic virus (TMV)

The following steps are taken:

1) Antibodies against the coat protein of TMV, intact virions or specific coat protein peptides and monoclonals are generated by hybridoma technology.
2) Hybridoma cell lines are cloned and cDNA sequences encoding the antibody heavy and light chains are cloned to generate a recombinant antibody or any recombinant version thereof. This is achieved using antibody heavy and light specific oligonucleotides and the reverse transcriptase polymerase chain reaction using isolated mRNA from a single hybridoma clone. This permits cloning of the full size antibody.
3) The cloned full size specific antibody heavy and light chain cDNAs from step 2 are used as a template for amplification of the heavy and light chain variable domains using specific oligonucleotide primers including a linker peptide sequence (i.e. GENEX 212 or $(Gly_4Ser)_n$) and splice overlap extension polymerase chain reaction. This step then provides the single chain antibody fragment and the two variable domains are linked by a 14 amino acid sequence.
4) The recombinant scFv gene from step 3 is inserted in a microbial or eukaryotic expression vector.
5) The binding specificity and function of the recombinant scFv (i.e. specificity and affinity for the target antigen) is checked after expression of the construct from step 4 in a heterologous host, such as in the periplasm of *E. coli*, using ELISA, surface plasmon resonance or western blotting.

6) A signal sequence is added to the 5' end of the recombinant scFv nucleotide sequence from step 3. A 3' linker peptide sequence (human T cell receptor constant domain) is added and this is then followed by the addition of a 3' transmembrane sequence from the human T cell receptor β chain. Suitable membrane localisation sequences also include the platelet derived growth factor receptor (PDGFR) transmembrane domain.

7) The 5' untranslated region from chalcone synthase is added to the 5' end of the construct from step 6.

8) The chimeric gene from step 7 is then inserted into a plant expression vector, such as pSS (Voss et al., 1995), upstream of the 3' untranslated region from Cauliflower mosaic virus, or any other source and the termination region from Cauliflower mosaic virus downstream of the 35SS promoter (Figure2). This vector also contains a selectable marker. In case of markerless and vectorless gene transfer selection marker sequences can be omitted.

9) *Agrobacterium tumefaciens* is transformed by N_2 transformation with the construct from step 8.

10) Expression and function of the recombinant scFv construct in plants are checked by transient expression in plant cells and ELISA, surface plasmon resonance or western blotting.

11) Transgenic plants are generated by transferring the construct from step 8, and a screenable selection marker, which is present in the pSS expression vector (e.g. the NPT-II gene for kanamycin resistance), into the plant genome by Agrobacteria mediated transformation.

12) Regenerated plants are screened using the selection marker for integration of the fusion gene.

13) Expression of the fusion protein in regenerated plants is followed by western blotting cell extracts, ELISA or surface plasmon resonance analysis.

14) The activity of the expressed fusion protein (i.e. affinity and specificity) is checked by ELISA using intact TMV virions as the antigen.

15) Localisation of the fusion protein is checked by indirect immuno-fluorescence, or confocal microscopy or immuno-electron microscopy.

16) The activity of the antibody in generating resistance against viruses is assayed by viral infection bioassays on transgenic plants, generated in steps 11 to 12 by using virions or infectious transcripts.

The orientation of Type II or tetraspan membrane protein can be exploited to permit display of molecular pathogenicdes to the cytoplasm after their synthesis in the secretory pathway. For cytoplasmic display of the recombinant scFv, steps 6) to 16) of example 1 are repeated with the following adaptations. The C-terminal membrane localisation sequence including the linker sequence and leader sequence of step 6 in example 1 are removed and a suitable linker and N-terminal targeting sequence belonging to the tetraspan family is added to the pathogen specific recombinant antibody to target and posttranslationally integrate recombinant proteins into the bilayer of plasma membranes. Suitable members of the tetraspan family include CD9, CD20, CD81 and the In-Hc-Ic dualspan typeII-IV hybrid of the MHC invariant chain and H-2^d hybrid protein. This method enables the orientation of a secreted and membrane anchored antibody construct with its N- and C-terminus into the cytosol.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised and cloned against structural and non structural proteins of any pathogen. Membrane anchor sequence(s) can be substituted against any sequence that facilitates membranes integration and provides a biological function. Moreover, example 1 can be combined with expression of any one of examples 2-8 in any combination(s) to give high level resistance to disease.

Construction of the scFv24 fusion expression cassette

To integrate the TMV-specific scFv24 into the plant cell membrane, the antibody fragment was fused to an N-terminal mammalian signal peptide and C-terminal receptor transmembrane domain. The mouse N-terminal light chain signal peptide from the parental antibody (mAb24) used to generate scFv24 was used to target fusion proteins to the secretory pathway. The transmembrane domain sequence of the human T-cell receptor β chain (TcRβ) was selected for fusion with the C-terminus of scFv24, for heterologous targeting of the scFv24 antibody to the plasma membrane. To ensure proper folding of the expressed single chain antibody fragments, the construct contained the constant region of TcRβ (pscFv24-TcRβ) as a linker sequence between the scFv24 fragment and the membrane anchor (Figure 7). The cloning of the neotope-specific anti-tobacco mosaic virus (anti-TMV) single-chain fragment scFv24CM including the leader peptide has been described (Zimmermann

et al. 1998). To generate the fusion construct pscFv24-TcRβ (Figures 2 and 7), a cDNA fragment encoding the constant and transmembrane domain of the human TcRβ chain (Yoshikai et al. Nature 312: 521-524 (1984)) was PCR amplified from human spleen mRNA (Clontech, Heidelberg, Germany) using the primers 5'- GCC *GTC GAC* GAG GAC CTG AAC AAG GTG TTC CCA - 3' (SEQ ID NO:1) and 5' – GCC *TCT AGA* TCA GAA ATC CTT TCT CTT G - 3' (SEQ ID NO:2). The primers contained restriction sites *Sal*I and *Xba*I (italics) to enable in frame cloning of the PCR product with scFv24CM (Zimmermann et al. 1998) resulting in the final construct scFv24-TcRβ.

For expression in plant cells, the *EcoR*I/*Xba*I fragment (Figure 7) of scFv24-TcRβ was subcloned into the *EcoR*I and *Xba*I restriction sites of the plant expression vector pSS (Voss et al. (1995)) containing the enhanced 35S promoter (Kay et al. (1987)) and the CaMV termination sequence (Figure 7, pscFv24-TcRβ).

Expression of the scFv24 fusion proteins in *N. tabacum* cv. BY-2 cell suspensions

To analyze the expression level of the recombinant scFv-fusion protein, the suspension cell line *N. tabacum* cv. BY-2 was stably transformed with recombinant *A. tumefaciens* and functional expression of the scFv24 domain of the fusion protein was analyzed by ELISA using anti-mAb24 antisera.

The vector construct pscFv24-TcRβ was transferred into *A. tumefaciens* GV3101 by liquid N_2 transformation (Höfgen and Willmitzer, Nucleic Acids Res. 16: 9877 (1988)). *N. tabacum* L. cv. bright yellow 2 (BY-2) cells were maintained in Murashige and Skoog basal salt with minimal organics (MSMO+: MSMO (Sigma, Deisenhofen, Germany) plus 200 mg/ml KH_2PO_4, 0.6 µg/ml thiamine, 3 % sucrose and 0.2 µg/ml 2,4-D, pH 5.8) at 24°C in the dark on an orbital shaker. Cells were subcultured every week with a 5 % inoculum. Three days after subculture, plant cells were transformed by co-cultivation with recombinant *A. tumefaciens*, as described (An, Plant Physiol. 79: 568-570 (1985)). Selection of kanamycin-resistant transformants was performed on MSMO+ agar medium supplemented with 75 µg/ml kanamycin and 100 µg/ml claforan.

For extraction of total soluble proteins from transgenic BY-2 suspension culture, cells from 1 ml culture were collected by centrifugation at 4000 x *g* for 5 min at 4°C. The cell pellet was resuspended in 1 ml protein extraction buffer (200 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02 % (w/v) sodium-azide and 0.1 % (v/v) Tween 20) and cells

were disrupted by sonication at 60 watt for 1 min using a sonicator probe (B. Braun, Melsungen, Germany) at 4°C. Cell debris was removed by centrifugation at 14000 x *g* for 10 min at 4°C. The clear supernatant containing soluble protein was used for further analysis.

For ELISA and western blotting, anti-mAb24 antisera (Zimmermann et al. (1998)) was used as a primary antibody in combination with a 1:5000 dilution of goat anti-rabbit alkaline phosphatase conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA). Protein concentrations were determined with the Bio-Rad Protein Assay using Bovine Serum Albumin (BSA) as standard.

Analysis of stably transformed *N. tabacum* BY-2 cells revealed that scFv24-TcRβ was completely intracellular (Figure 8). scFv24-TcRβ was not detectable in the culture supernatant, indicating that the TcRβ transmembrane domain is stable and suitable for targeting scFv24 to tobacco cell membranes.

Characterization of transgenic plants

It was then tested whether the heterologous mammalian transmembrane domain TCRβ fused to scFv24 would target the single chain antibody to the plasma membrane in stably transformed tobacco plants. Transgenic *N. tabacum* cv. Petite Havana SR1 were generated by the leaf disc transformation with recombinant *A. tumefaciens* and transgenic T_0 plants were generated from transformed callus (Horsch et al., Science 227: 1229-1231 (1985)). Extraction of total soluble proteins from tobacco leaves and subsequent analysis of scFv24 by ELISA were performed as described by Fischer *et al.* [Fischer R, Drossard J, Liao YC, Schillberg S: Characterisation and applications of plant-derived recombinant antibodies. In: Cunningham C, Porter AJR (eds), Recombinant proteins in plants: Production and Isolation of Clinically useful compounds, pp. 45-68. Vol. 3. Humana press, Totowa, NJ (1998)]. For ELISA and western blotting, anti-mAb24 antisera (Zimmermann et al. (1998)) was used as a primary antibody in combination with a 1:5000 dilution of goat anti-rabbit alkaline phosphatase conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA). Protein concentrations were determined with the Bio-Rad Protein Assay using Bovine Serum Albumin (BSA) as standard.

Expression levels of scFv24-TcRβ were much higher in transgenic *N. tabacum* cv. Petite Havana SR1 plants than in suspension cultures (Table 1). The maximum level of detergent extracted scFv24-TcRβ was 296 fold higher (8866 ng/g leaf tissue) than

that obtained in transgenic suspension cultures (Figure 8).

To determine if the scFv24 fragment was stably integrated into the plasma membrane or secreted into the extracellular space of intact plants, intercellular washing fluid from leaves of transgenic T_1 tobacco plants was analyzed by ELISA. For detection of scFv24 fusion protein in intercellular washing fluids, leaves of *N. tabacum* cv. Petite Havana SR1 were prepared as described by Fischer *et al.* (Fischer: Characterisation and applications of plant-derived recombinant antibodies. In: Cunningham C, Porter AJR (eds), Recombinant proteins in plants: Production and Isolation of Clinically useful compounds, pp. 45-68. Vol. 3. Humana press, Totowa, NJ (1998)). Total protein extracts from washing fluids were concentrated by ultrafiltration (Microcon 10, Amicon, Witten, Germany) and analyzed by 12% SDS-PAGE (Laemmli, Nature 227: 680-685 (1970)) followed by western blot. There was no detectable antibody in the intercellular washing fluid from ten T_1 progenies of two plant lines ($T4_{SR1}$ and $T6_{SR1}$) producing the scFv24-TcRβ fusion protein. In general, T_1 plants used for IWF analysis showed high expression levels of intracellular scFv24-TcRβ (1570-8940 ng/g leaf tissue).

Western blot analysis of total soluble protein isolated from a T_1 progeny of plant line $T4_{SR1}$ showed only the predicted full length 48.2 kDa scFv24-TcRβ fusion protein and neither the intact fusion protein nor any degradation products were detectable in the intercellular washing fluid (Figure 9). This demonstrates that scFv24-TcRβ was not secreted and remained membrane anchored in transgenic plants.

Subcellular localization of scFv24-TcRβ in transgenic *N. tabacum* cv. BY-2 protoplasts

Since the scFv24-TcRβ construct contains signal peptide and transmembrane sequences, fusion protein should be localised at the plasma membrane. To determine the subcellular localization of the scFv24-fusion protein, transgenic *N. tabacum* cv. BY-2 protoplasts were generated and analysed by immunofluorescence microscopy (Figure 10).

Protoplasts were prepared by digesting 3 day old tobacco BY-2 cells with 1.5 % (w/v) cellulase Onozuka RS (Yakult Honsha Co., Tokyo, Japan), 0.7 % (w/v) hemicellulase (Sigma) and 0.1 % (w/v) pectolyase Y23 (Seishin Pharmaceuticals, Nihonbashi, Tokyo, Japan) in MES buffer (0.5 % (w/v) MES, 80 mM $CaCl_2$, 0.3 M mannitol, pH 5.8) for 1.5 h at 25°C on a rotary shaker. Protoplasts were washed with MES buffer,

transferred to fresh MSMO+ medium and incubated over night at 24°C on an orbital shaker in the dark. Regenerating protoplasts were washed once with MES buffer and settled on poly-L-lysine-coated multiwell slides. Cells were fixed for 15 min at room temperature with 4 % (w/v) formaldehyde in MTS buffer (50 mM Pipes, 5 mM EGTA, 5 mM Mg_2SO_4, pH 6.9) plus 0.3 M mannitol. The resulting protoplast ghosts were washed with MTS buffer and incubated for 1 h at room temperature with a 1:2500 dilution of rabbit anti-mAb24 antisera (Zimmermann et al. (1998)) or a 1:50 dilution of mouse anti-human TcR β IgG (T Cell Diagnostics, Woburn, MA) in 3 % (w/v) BSA MTS buffer supplemented with 0.3 M mannitol. Cells were washed with MTS buffer and then incubated for 1 h at room temperature with FITC-conjugated goat anti-rabbit or FITC-conjugated goat anti-mouse secondary antibodies (Jackson Immuno Research Laboratories) diluted 1:100 in 3 % (w/v) BSA MTS buffer supplemented with 0.3 M mannitol. After washing with MTS buffer, slides were mounted in Citifluor antifade (Citifluor Ltd., London, England) and imaged on a Zeiss inverted microscope equipped with a 40x oil-immersion objective using 450-490 nm excitation and 520-560 nm emission interference filters. Images were recorded on T-max 400pro film (Kodak, Rochester, NY).

Protoplasting enzymes contain proteases which degrade cell surface proteins and there was no detectable cell surface staining directly after protoplasting BY-2 cells. However, overnight regeneration of protoplasts allowed delivery of newly synthesized membrane bound scFv24 to the cell surface and gave optimal staining. Control wild type *N. tabacum* cv. BY-2 cells showed no evidence of surface staining (Figure 10A). In contrast, protoplasts derived from the transgenic *N. tabacum* cv. BY-2 suspension cell line $T2_{BY-2}$ producing scFv24-TcRβ were brightly stained with anti-Ab24 sera at the plasma membrane, demonstrating the plasma membrane localization of the scFv24-TcRβ fusion protein (Figure 10B). In addition, the localisation of scFv24-TcRβ to the plasma membrane was also observed by staining with the anti-human TcRβ antibody, which recognizes the constant region of TcRβ that links the TcRβ transmembrane domain to scFv24 (Figure 10C). In addition to plasma membrane labeling, some protoplasts showed nuclear membrane staining for the scFv24-TcRβ fusion protein (Figure 10D).

Immuno-electron microscopy

Immuno-electron microscopy was performed on ultrathin section of leaves, to verify

location of membrane bound scFv24.

Small tissue pieces of transgenic *N. tabacum* cv. Petite Havana SR1 plants were embedded at low temperature for immunogold labeling (Wells, Micron and Microscopica Acta 16: 49-53 (1985)). Immunogold labeling of thin sections on plastic-filmed gold grids was carried out as previously described (McCann et al. J. Microsc. 166: 123-136 (1992)), except that blocking buffer used for the incubations with antibodies contained 3 % (w/v) BSA. Primary antibodies: rabbit anti-mAb24 antiserum at 1:100 dilution (Zimmermann et al. (1998)) or mouse anti-human TcRβ IgG at 1:25 dilution (T Cell Diagnostics), were incubated with sections for 1 h. Secondary antibodies were 12 nm gold conjugated goat anti-rabbit or 12 nm gold conjugated goat anti-mouse (Jackson Immuno Research Laboratories) and were incubated with sections at a dilution of 1:40 for 1 h. Bright-field light micrographs of 1 μm thick resin sections were viewed at 80 kV on a Joel 1200EX transmission electron microscope, and photographs were taken using Kodak film.

In young leaves of the transgenic T_0 plant $T4_{SR1}$ producing scFv24-TcRβ, scFv24 was localized to both the nuclear and plasma membranes (Figure 11), confirming the subcellular localization of scFv24-TcRβ by immunofluorescence in transgenic *N. tabacum* cv. BY-2 protoplasts. The number of gold particles found in chloroplasts, mitochondria, glyoxysomes, cytoplasmic matrices and vacuoles was consistent with background labeling.

Bioassays of viral resistance

To analyze the biological effects of the membrane anchored anti-viral TMV-specific antibody on viral resistance, T_1 progenies of plant line expressing the scFv24-TcRβ fusion protein ($T6_{SR1}$) were inoculated with TMV.

Seeds were collected from antibody-producing T_0 plants and germinated on MSMO agar medium supplemented with 2 % (w/v) sucrose, 0.4 μg/ml thiamine, 0.4 μg/ml glycine, 0.1 μg/ml nicotine acid, 0.1 μg/ml pyridoxine and 75 μg/ml kanamycin. Kanamycin-resistant T_1 plants were used for inoculation with TMV-*v* (1 μg/ml) as previously described (Dietzgen, Arch. Virol. 87: 73-86 (1986)). Wild type *N. tabacum* cv. Petite Havana SR1 plants were used as a control. Disease symptoms were monitored 6 to 20 days post inoculation (p.i.) and for resistant plants up to 180 days p.i..

Lower leaves were infected with TMV and systemic spread of the virus was followed

by analyzing upper leaves 6-20 days later. All control non-transgenic tobacco plants were systemically infected, but 16 % (out of 31 analyzed) of scFv24-TcRβ transgenic plants had no visible disease symptoms on the upper leaves (Table 2). Furthermore, ELISA analysis demonstrated that some of these plants accumulated virus particles in the upper leaves indicating that though systemic viral spread occurred, no symptoms were developed. Strikingly, in 6 % of scFv24-TcRβ transgenic plants no virus was found in the upper leaves up to 90 days post inoculation. Virus could be detected at inoculation sites in the lower leaves by ELISA demonstrating that these plants had been efficiently inoculated with TMV. Antibody-fusion protein expression levels correlated with expression of TMV resistance (Table 2). Higher levels of scFv24 fusion protein expression led to an increased fraction of virus resistant plants, confirming that membrane anchored scFvs could be used to generate plants resistant to virus.

Conclusions

The recombinant fusion protein scFv24-TcRβ was functionally expressed in transgenic tobacco suspension cultures and transgenic plants. Expressed scFv24-TcR recognized TMV in ELISA and showed the expected size in immoblot analysis. Furthermore, immunofluorescence and electron microscopy showed that the TcR• transmembrane domain targeted scFv24 to the tobacco plasma and nuclear membrane. Bioassays of viral infection showed that transgenic tobacco plants expressing scFv24-TcR• were resistant to systemic TMV infection. These results demonstrated that membrane anchored anti-viral antibody fragments are functional, can be targeted to the plasma membrane *in planta* and are a novel method to shield plant cells from invading pathogens.

Table 1:

Levels of functional scFv24 fusion protein in the T_0 generation of transgenic *N. tabacum* cv. Petite Havanna SR1.

Total soluble plant protein was isolated from leaves of transgenic plants producing scFv24-TcRβ. scFv24-fusion protein expression was quantified by TMV-specific ELISA using anti-mAb24 antisera and expressed as ng scFv24 per g leaf tissue.

Construct	Number of transgenic plants	Number of plants expressing functional scFv24	Range of expression (ng/g leaf tissue)	Average expression (ng/g leaf tissue)
pscFv24-TcRβ	6	6	30 - 8866	1991

Table 2:

Virus infection assay of trangenic plants expressing membrane anchored scFv24.

1 µg/ml TMV-*v* was applied onto a lower leaf of non-transgenic *N. tabacum* cv. Petite Havana SR1 and transgenic T_1 progenies from plant line $P9_{SR1}$ producing scFv24-PDGFR or $T6_{SR1}$ producing scFv24-TcRβ. scFv24-fusion protein levels were determined by ELISA using the anti-mAb24 antisera 14 days p.i. and used for group formation (low, average and high producers). [a] = upper leaves showed no visible disease symptoms; [b] = based on TMV-ELISA; [c] = level of resistance of all low, average and high producers, numbers in brackets include all plants without visible disease symptoms.

Plant lines	Tested plants	ng scFv24 per g leaf tissue	Healthy phenotype[a]	Resistant plants[b]	Level of resistance (%)[c]
N. tabacum cv. Petite Havana SR1	62	-	0	0	0
$T6_{SR1}$, low producer	22	10-500	1	0	
$T6_{SR1}$, average producer	2	501-2000	2	1	6 (16)
$T6_{SR1}$, high producer	7	2001-21500	2	1	

Example 2: Expression of a neutralising anti-viral antibody with a C-terminal membrane localisation sequence

Cytoplasmic presentation of a membrane localised recombinant antibody against the coat protein of Tobacco Mosaic virus (TMV)

The steps 1) to 16) of example 1 are repeated with the following adaptations.

1) The N-terminal signal sequence is removed and replaced by a start codon.
2) The C-terminal membrane localisation sequence including the linker sequence of example 1 are replaced by suitable linker and C-terminal targeting sequences to posttranslationally target and integrate recombinant proteins into the bilayer of endomembranes. Suitable targeting sequences include transmembrane domains of KAR1 for nuclear membrane integration (Rose and Fink, 1987), middle-T antigen for plasma membrane integration (Kim et al., 1997) and cytochrome b5 for ER membrane integration (Kim et al., 1997). Moreover, prenylation, farnesylation, palmitoylation, myristoylation and ankyrin sequence motifs can be incorporated.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised against structural and non structural proteins of any pathogen. Membrane anchor sequence(s) can be substituted against any sequence that facilitates membranes integration and provides a biological function. Moreover, example 2 can be combined with examples 1 and 3-8 in any combination(s).

Construction of the scFv24 fusion expression cassette

To integrate the TMV-specific scFv24 into the bilayer of endomembranes the antibody fragment was fused to a C-terminal receptor transmembrane domain. No N-terminal signal sequence was included, therefore the scFv fragment is facing to the cytosol. The transmembrane domain sequence of KAR1 was selected for nuclear membrane integration (Rose and Fink, 1987), middle-T antigen for plasma membrane integration (Kim et al., 1997), cytochrome b5 for ER membrane integration (Kim et al., 1997) and syn1 for integration into vesicles (Kim et al., 1997). To ensure proper folding of the expressed single chain antibody fragments, the constructs scFv24-kar1, scFv24-mT, scFv24-cytb5 and scFv24-syn1 contained a Gly_4Ser linker sequence between the scFv24 fragment and the membrane anchor

(Figure 12).

The C-terminal transmembrane domains KAR1, middle-T (mT), cytochrome b5 (cytb5) and synaptobrevin 1 (syn1) including the Gly_4Ser linker sequence were fused to the scFv24 by PCR. The template scFv24CW cDNA (Zimmermann et al. (1998)), the forward primer –40 (5'-GTT TTC CCA GTC ACG AC-3' (SEQ ID NO:4)) and the following backward primers were used for PCR amplification: for kar1 5'-GGC TCT AGA CGC TCG AGT TTA AAA CCT ATA ATA CAC ATA GAT GTT GCA ATA AAG CAA AAT CAG TAT ACA AAT AGT CCA CCA GAA ATA CTC CCT ATA CTT CTT AGC GGC CGC AGA ACC TCC ACC TCC GTC G-3' (SEQ ID NO:5); for mT 5'-GGC TCT AGA CGC TCG AGT TTA GAA ATG CCT AGA TCT CTT AAT CAA GAT GAA GAG CAT CAA GCA AAT TCC GAG CAG CGC TGC CAA GAA AGT CAC CAA GAG CAA AGT TCT TCC CAA TCT CCT AGC GGC CGC AGA ACC TCC ACC TCC GTC G-3' (SEQ ID NO:6); for cytb5 5'-GGC TCT AGA CGC TCG AGT TTA ATC CTC TGC CAT GTA GAG TCT ATA CAT GAG AGC AAC CAC GAG TGC TGA TAT CGC TGG GAT CAC CCA ATT GGT CCA CCA TGA AGA GTT AGA CTC AAC AGC GGC CGC AGA ACC TCC ACC TCC GTC G-3' (SEQ ID NO:7) and for syn1 5'-GGC TCT AGA CGC TCG AGT TTA AGT GAA GAA ATA AAT AAC AAT AAC AAC AAC AAT AAT AGC ACA AAT AGC ACC AAG CAT AAT CAT CAT CTT ACA ATT CTT CCA AGC GGC CGC AGA ACC TCC ACC TCC GTC G-3'(SEQ ID NO:8). The codons of the transmembrane domains were codon optimized for expression in tobacco, pea and wheat. The 5'-*Eco*RI and 3'-*Xba*I restricted PCR fragments were subcloned into pUC18 and sequenced. Mutations in the mT and syn1 transmembrane domain were eliminated by PCR using the forward primer –40 (5'-GTT TTC CCA GTC ACG AC-3' (SEQ ID NO:9)) and the backward primers MUT7MT 5'-GGC TCT AGA CGC TCG AGT TTA GAA ATG CCT AGA TC-3' (SEQ ID NO:10) for mT and SYN1SHORT 5'-GGC TCT AGA CGC TCG AGT TTA AGT GAA GAA ATA AAT AAC AAT AAC AAC AAC-3' (SEQ ID NO:11) for syn1. The chalcone synthase 5' untranslated region and scFv24 was substituted by the *Eco*RI and *Sal*I fragment from scFv24CW (Zimmermann et al. (1998)). The subsequent ligation of the *Eco*RI-*Xba*I fragments into the plant expression vector pSS, containing an double enhanced 35S promoter (Voss et al. (1995)), resulted in the final expression constructs pscFv24-kar1, pscFv24-mT, pscFv24-cytb5 and pscFv24-syn1 (Figure 12).

Transient expression in tobacco leaves

To analyze the expression level of the recombinant scFv-fusion proteins, *N. tabacum* cv. Petite Havana SR1 leaves were transiently transformed with recombinant *A. tumefaciens* and functional expression of the scFv24 domain of the fusion protein was analyzed by ELISA using anti-mAb24 antisera.

The vector constructs were transferred into *A. tumefaciens* GV3101 by liquid N_2 transformation (Höfgen and Willmitzer (1988)). Transient transformation of *N. tabacum* cv. Petite Havana SR1 was performed as described (Kapila et al., Plant Science 122 (1996) 101-108). To extract total soluble proteins, Tobacco leaves were frozen and ground in liquid nitrogen and scFv-fusion protein level was analysed by ELISA using anti-mAb24 antisera (Zimmermann et al. (1998)) as a primary antibody in combination with a 1:5000 dilution of goat anti-rabbit alkaline phosphatase conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA) (Fischer et al. in: C. Cunningham, A.J.R. Porter, (Eds.), Methods in biotechnology Vol. 3: Recombinant proteins in plants: Production and Isolation of Clinically useful compounds. Humana Press, Totowa, NJ (1998), ELISA III).

The fusion proteins scFv24-mT, scFv24-syn1 and the control scFv24 lacking a C-terminal transmembrane domain were not detectable in plant extracts from transient transformation experiments (detection limit: 0.5 ng per gram leaf tissue). However, scFv24-kar1 and scFv24-cytb5 accumulated in the cytosol of plant cells to a maximum level of 1.8 ng and 1.0 ng per gram leaf tissue, respectively.

Conclusions

Generation of stable transformed *N. tabacum* cv. Petite Havana SR1 and bioassays to analyse the biological effects of cytosolic scFv24-kar1 and scFv24-cytb5 in transgenic tobacco plants are in progress. Based on our results with the scFv24-TcRβ (example 1) integration of the fusion proteins into the plant cell membrane was expected via the C-terminal transmembrane domains KAR1 or syn1. The scFv24 is facing to the plant cytosol, which will result in an increased viral resistance because the biological effect of scFv24 is high in the plant cytosol and membrane integration targets the scFv24 to the localisation where the virus is most vulnerable. The virus replication and movement takes place at plant cell membrane systems, therefore virus dissambly will be effectively prevented.

Example 3: Viral resistance by expression of a molecular pathogenicide

Fusion of a nuclease activity to a recombinant antibody specific for TMV

The steps 1) to 16) of example 1 and/or the steps 1) to 2) of example 2 are repeated with the following adaptations.

1) The plant expression construct contains a 5' signal sequence to enable delivery of the recombinant antibody to the ER lumen and then secretion to the apoplast.
2) The transmembrane targeting domain is replaced by linker coupling the protein to a C-terminal fusion with a toxin – in this case an RNAse enzyme which degrades cellular RNA, viral RNA, replicative forms and/or replicative intermediates.
3) Upon binding to the virions in the apoplast, the fusion protein will enter the cytosol of damaged cells via the entering virions, where the cytotoxic RNase will degrade viral RNA, replicative intermediates and replicative forms or/and cellular RNA and cause cell death of virally infected cells and therefore prevent replication and spread of the pathogen.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised against structural and non structural proteins of any pathogen. The RNase sequence(s) can be substituted against any enzyme sequence that interferes in the pathogen life cycle. Moreover, example 3 can be combined with examples 1-2 and 4-8 in any combination(s).

Construction of the scFv24 fusion (immunotoxin) expression cassette

To generate an apoplastic expressed immunotoxin, the TMV-specific scFv24 was fused to an N-terminal mammalian signal peptide and C-terminal toxin. The mouse N-terminal light chain signal peptide from the original antibody (mAb24) used to generate scFv24 was used to target fusion proteins to the secretory pathway. The domain III of the *Pseudomonas* exotoxin (PE) was selected for fusion with the C-terminus of scFv24. The domain III of the *Pseudomonas* exotoxin mediates the ADP-ribosylation of elongation factor 2, which arrests protein synthesis and causes cell death. To ensure proper folding of the expressed single chain antibody fragments, the cDNA construct contained the cellobiohydrolase I (CBHI) linker of *Trichoderma reesi* (Mallender and Voss, 1994, J. Biol. Chem. 269, 199-206) between the scFv24 fragment and the domain III of the PE. A 12 amino acid residue epitope tag (tag54)

was fused to the C-terminus via a Gly_4Ser linker to enable detection of the recombinant protein in plant extracts.

The PE domain III was PCR amplified from the plasmid PE38 (Theuer et al., Cancer Res 15, 340-347 (1993)) using the primers PE400-for 5'- GCG GAA TTC GAC GTC GCC ATG GCC TTC CTC GGC GAC GGC GGC GAC - 3' (SEQ ID NO:16) and PE-back 5'- GCG AAG CTT GTC GAC CGG CGG TTT GCC GGG CTG GCT G - 3' (SEQ ID NO:17). The primers contained restriction sites *Eco*RI, *Aat*II and *Nco*I (PE400-for) and *Sal*I and *Hind*III (PE-back) for cloning. The PCR fragment was subcloned via *Eco*RI and *Hind*III into pUC18 resulting in the construct PE400-intermediate and the sequence was verified by sequencing. To generate the immunotoxin, the scFv29 sequence from the biscFv2429-apoplast in pUC18 (Fischer et al., *Eur. J. Biochem.* 262, 810-816 (1999)) was removed by *Aat*II and *Sal*I and exchanged by the *Aat*II (internal restriction site) and *Sal*I fragment of PE400-intermediate. Finally, the *Eco*RI and *Sal*I fragment containing the chalcone synthase 5' untranslated region, the leader signal of mAb24 light chain (Voss et al., 1995), the scFv24, the CBHI linker and PE domain III was subcloned into the *EcoR*I and *Sal*I restriction sites of pSS derivate containing the enhanced 35S promoter (Kay et al., 1987), the epitope tag54 and the CaMV termination sequence resulting in the final plant expression construct pscFv24-PE400 (Figure 13).

Conclusions

Generation of stable transformed *N. tabacum* cv. Petite Havana SR1 and bioassays to analyse the biological effects of scFv24-PE400 in transgenic tobacco plants are in progress. However, PE280-tag29 was transiently expressed in tobacco leaves and a slightly degraded product of was detected in plant extracts of transient transformed tobacco leaves, indicating that PE280-tag29 is most likely expressed and secreted to the apoplast and therefore not toxic to the plant cells (see example 7). Consequently, expression of the immunotoxin scFv24-PE400 will be non-toxic to plants.

The scFv24-PE fusion (scFv24-PE400) will be secreted to the apoplast of transgenic tobacco plants. During TMV infection the fusion protein will bind to the virus particle via the scFv24 (as shown for the full-size rAb24) and TMV virions that enter the cell will carry bound scFv24-toxin fusion. PE400 mediates the ADP-ribosylation of elongation factor 2, which arrests protein synthesis and causes cell death. PE is a very effective toxin, as only a few molecules are required to kill the infected cell, thus

preventing virus replication and spread, leading to highly resistant plants.

Molecular Pathogenicide fusion (RNAse fusion)

A C- or N-terminal fusion of a coat protein specific antibody scFv gene (scFv24) with a cDNA encoding a RNAse gene (for example *E. coli* RNAse E) results in an scFv-enzyme fusion protein which can be targeted to the plant cytoplasm - or to cellular organelles, membranes, or the apoplast to interfere in viral replication.

Such an scFv-RNAse fusion was engineered based on the TMV-specific scFv24 which binds to the intact TMV virions instead of coat protein alone. This scFv24 was chosen for targeting the viral RNA at the earliest timepoint of the viral infection cycle to immediately act on the released viral RNA upon viral dissassembly. A second contruct is based on a 30K specific scFv-RNAse fusion which follows the same set up as given in Fig 14 based on the scFv24. This scFv is described in Example 5 (scFv 30-1 or scFv 30-2) and will be coexpressed with the scFv 24-RNaseE fusion in double transgenic plants transformed with both constructs. For fusion to the above mentioned scFv antibodies the *E. coli* RNAse E. was selected (Claverie-Martin et al., *J. Biol. Chem.* 266, 2843-2851] and connected to the scFv upon PCR-amplification using standard cloning technologies known by any person skilled in the art.

Example 4: Enhanced coat protein mediated resistance with an antibody-viral coat protein fusion protein

Fusion of a viral coat protein to a recombinant antibody specific for TMV

The steps 1) to 16) of example 1 and/or the steps 1) to 2) of example 2 are repeated with the following adaptations.

1) The transmembrane targeting domain listed in example 1 are removed but the C-terminal anchor and linker sequences of example 2 can be maintained.
2) The N-terminal signal sequence of example 1 is replaced by an upstream located (N-terminal) TMV coat protein monomer and then connected via a flexible linker to the recombinant antibody cDNA.
3) The fusion protein is expressed in the cytosol.
4) Alternatively, the transmembrane domain is replaced by a linker enabling C-

terminal fusion with the TMV coat protein monomer. The fusion protein is expressed in the cytosol (without N-terminal signal sequence) or sent into the secretory pathway via a N-terminal signal peptide.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised against structural and non structural proteins of any pathogen. The N-terminal coat protein sequence(s) can be substituted against any sequence (for example Glutathione S-Transferase, Thioredoxin, plant virus movement proteins, replicase, minimal proteins or domains thereof) that stabilises a cytosolic expressed recombinant antibody and interferes in the pathogen life cycle. Moreover, example 4 can be combined with examples 1-3 and 5-8 in any combination(s).

Construction of the scFv24 fusion expression cassettes

A cytoplasmically expressed protein (the coat protein of tobacco mosaic virus) was selected as fusion partner to analyse its effect on the function and stability of the TMV-specific scFv24. The tobacco mosaic virus coat protein (CP) was cloned 5' upstream of the scFv24 (Figure 15).

The gene fusion partner coat protein (CP) from TMV was amplified by PCR. cDNAs was amplified from a cDNA clone from TMV as a template. The forward primers introduced a *Nco*I restriction site (5' end) and the backward primers a C-terminal $(Gly_4Ser)_2$ linker sequence and an *Aat*II restriction site (3' end). The following forward and backward primers were used for PCR amplification: CP-for 5'-ACT GCG CCA TGG CTT ACA GTA TCA CT-3' (SEQ ID NO:20), CP-back 5'-CCG TCA GAC GTC AGA ACC TCC ACC TCC ACT TCC GCC GCC TCC AGT TGC AGG ACC AGA GGT CCA AAC CAA ACC-3' (SEQ ID NO:21). The 5'-*Nco*I and 3'-*Aat*II restricted PCR fragments were subcloned into a pUC18 derivative containing the TMV specific scFv24 (Zimmermann et al., 1998) flanked by the 5' Ω untranslated region (omega-sequence) and 3' untranslated region (Pw sequence) from TMV (Schmitz et al., Nucleic Acids Res 24 (1996) 257-263); (Gallie and Kobayashi, Gene 142 (1994) 159-165). A C-terminal His6- (H) or KDEL-sequence (K) were added to scFv24 of the fusion construct by PCR using the backward primers: His6-back 5'-CTA CCC CTC GAG TTT AGT GAT GGT GAT GGT GAT GAG CGG CCG CGT CGA CTG CAG

AGA CAG TGA CCA GAG TC-3' (SEQ ID NO:22) and KDEL-back 5'-CCC TCA CTC GAG TTT AGA GCT CAT CTT TCT CAG ATC CAC GAG CGG CCG CAG AAC CTC CAC CTC CGT CGA CTG CAG AGA CAG TGA CCA G-3' (SEQ ID NO:23). The subsequent ligation of the *Eco*RI-*Asc*I fragments into the plant expression vector pSS, containing an double enhanced 35S promoter (Voss et al., 1995), resulted in the final expression constructs CP-scFv24H and CP-scFv24K, which were used for analyzing scFv-fusion protein accumulation in the cytoplasm (Figure 15A).

For ER targeting and retention, the plant codon optimized leader sequence derived from the light chain of the murine monoclonal antibody mAb24 (Voss et al., 1995) was integrated between the 5' Ω untranslated region and scFv24 of the cytoplasmic construct containing the KDEL sequence, giving the plant expression vector L-CP-scFv24K (Figure 15C). For targeting the same construct to the apoplast, the c-terminal KDEL sequence was replaced by a His 6 sequence, giving the plant expression vector L-CP-scFv24H (Figure 15D).

Two expression vectors lacking a leader sequence and an N-terminal fusion partner but containing scFv24 with a C-terminal His6 or KDEL sequence were used as controls for cytoplasmic accumulation (scFv24H, scFv24K, Figure 15B).

Analysis of fusion protein accumulation in the ER

Accumulation of functional fusion protein in the ER was analysed by transient expression in *N. tabacum* cv. Petite Havana SR1 leaves and functional scFv24 was detected by a TMV-specific ELISA. Plant expression constructs were transferred into *A. tumefaciens* GV3101 by N_2 transformation (Höfgen and Willmitzer, Nucleic Acids Res 16 (1988) 9877). Transient transformation of *N. tabacum* cv. Petite Havana SR1 was performed as described (Kapila et al., Plant Science 122 (1996) 101-108). To extract total soluble proteins, Tobacco leaves were frozen and ground in liquid nitrogen and scFv-fusion protein level was analysed by ELISA and Western blot (Fischer et al., Methods in biotechnology Vol. 3: Recombinant proteins in plants: Production and Isolation of Clinically useful compounds. Humana Press, Totowa, NJ (1998), ELISA III). A Fab fragment of the mAb24 was used as the ELISA standard. Protein concentrations were determined with the BioRad Protein Assay using bovine serum albumin (BSA) as the standard.

The level of functional scFv24 detected for the ER retained L-CP-scFv24K fusion protein, amounted to 1μg per gram leaf material (average 0.6μg per gram leaf

material, Figure 16).

To verify the integrity of scFv24 fusion proteins, western blot analysis was carried out using affinity purified L-CP-scFv24K. For affinity purification of scFv-fusion proteins from plant extracts (prepared as described above), TMV virions were coupled to an activated CNBr sepharose matrix. 300mg of CNBr activated sepharose 4B matrix (Pharmacia, Freiburg, Germany) was resuspended in 1ml PBS pH 7.4 (1.37M NaCl, 27mM KCl, 81mM Na_2HPO_4, 15mM KH_2PO_4) and incubated for 1h at RT on a rotator. The matrix was pelleted (5000xg, 5min, RT), resuspended in 1ml PBS pH7.4 containing 10mg TMV virions and incubated for 2h at RT on a rotator. The TMV coupled matrix was centrifuged (5000xg, 5min, RT), resuspended in 1ml PBS pH 7.4 containing 1% (w/v) BSA and 1% (w/v) powdered milk and rotated over night at 8°C to block nonspecific binding sites. The TMV coupled matrix was washed three times with PBS pH 7.4 and resuspended in 1ml PBS pH 7.4. 30μl TMV-matrix was added to 1.5ml plant extract (prepared as described above) and incubated for 1h at RT on a rotator. Then the TMV-matrix was washed three times with PBS and the TMV-matrix bound proteins were solubilised in sample buffer and analysed by SDS-PAGE (Laemmli, Nature 227 (1970) 680-685) and Coomassie brillant blue staining.

Intact fusion proteins were detected with the expected size of 49.5kDa for L-CP-scFv24K (Figure 17).

Analysis of fusion protein accumulation in the cytoplasm

For transient cytoplasmic accumulation of fusion proteins *N. benthamiana* leaves were used. The vector constructs were transferred into *A. tumefaciens* GV3101 by liquid N_2 transformation (Höfgen and Willmitzer, Nucleic Acids Res. 16: 9877 (1988)). Transient transformation of *N. benthamiana* was performed as described (Kapila et al., Plant Science 122 (1996) 101-108). To extract total soluble proteins, Tobacco leaves were frozen and ground in liquid nitrogen and scFv-fusion protein level was analysed by ELISA and Western blot (Fischer et al., Methods in biotechnology Vol. 3: Recombinant proteins in plants: Production and Isolation of Clinically useful compounds. Humana Press, Totowa, NJ (1998), ELISA III). A Fab fragment of the mAb24 was used as the ELISA standard.

Analysis using the constructs containing the C-terminal His6 sequence (Figure 15) demonstrated that CP-scFv24H was detectable in a TMV-specific ELISA, with an average protein level of 0.9ng functional active scFv24 per gram leaf material.

Protein levels of the control construct scFv24H lacking an N-terminal fusion partner was below the ELISA detection limit (0.5ng) (Figure 18).

We evaluated the influence of a C-terminal KDEL sequence on the accumulation of scFv24 fusion proteins. Addition of the C-terminal KDEL sequence increased the level of the fusion protein (Figure 18). The average protein level of the KDEL tagged CP-scFv24K was 3fold higher than CP-scFv24H (2.9ng per gram leaf material). Level of the control construct scFv24K was below the ELISA detection threshold. A control ELISA performed without the antigen TMV gave no signal, indicating that values of CP-scFv24H and CP-scFv24K could not be correlated with specific binding of CP-fusions to anti TMV polyclonal sera.

Characterization of transgenic plants expressing cytoplasmic scFv fusion proteins

We then tested to which level the cytoplasmically expressed CP-scFv24K accumulated in stably transformed tobacco plants. Transgenic *N. tabacum* cv. Petite Havana SR1 were generated by the leaf disc transformation with recombinant *A. tumefaciens* and transgenic T_0 plants were generated from transformed callus (Horsch et al., Science 227: 1229-1231 (1985)). Extraction of total soluble proteins from tobacco leaves and subsequent analysis of scFv24 by ELISA were performed as described by Fischer *et al.* (Fischer et al., In: Cunningham C, Porter AJR (eds), Recombinant proteins in plants: Production and Isolation of Clinically useful compounds, pp. 45-68. Vol. 3. Humana press, Totowa, NJ (1998)). For ELISA anti-mAb24 antisera (Zimmermann et al., Molecular Breeding 4: 369-379 (1998)) was used as a primary antibody in combination with a 1:5000 dilution of goat anti-rabbit alkaline phosphatase conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA) (Fischer, Methods in biotechnology Vol. 3: Recombinant proteins in plants: Production and Isolation of Clinically useful compounds. Humana Press, Totowa, NJ (1998), ELISA III).

Protein levels of CP-scFv24K showed an average of 1.2 ng per gram leaf material in 12 analysed transgenic *N. tabacum* cv. Petite Havana SR1 plants. The maximum level of detergent extracted CP-scFv24K was 2.3 ng per g leaf tissue, thus higher than the scFv24 without fusion partner and KDEL sequence (maximum 1.8 ng/g leaf tissue; average 0.8 ng/g leaf tissue; Zimmermann et al., Molecular Breeding 4 (1998).

Conclusions

Bioassays to analyse the biological effects of cytosolic CP-scFv24K in transgenic *N. tabacum* cv. Petite Havana SR1 plants are in progress. Based on other inoculation experiments, an increase of viral resistance can be expected when compared to cytosolic scFv24 without fusion partner which creates a resistant phenotype, based on the increase of scFv24 protein levels and the presence of TMV-CP and TMV-RNA sequences to induce CP-mediated and RNA mediated virus resistance in addition to the primary antibody resistance.

Example 5: Enhanced resistance by the expression of an anti-viral movement protein antibody

Expression of recombinant antibodies against the TMV 30K movement protein in transgenic tobacco

The steps 1) to 16) of example 1 and/or the steps 1) to 2) of example 2 are repeated with the following adaptations.

1) Specific antibodies recognising the TMV 30K movement protein are raised by hybridoma technology, phage or ribosome display screening and subsequently cloned to engineer single chain antibodies or any recombinant form thereof.
2) The antibody is expressed in the cytosol or sent into the secretory pathway or membrane localised. The recombinant antibody may cause the desired biological effect without a fusion partner so the toxin sequence may be omitted.
3) For ELISA and surface plasmon resonance the test antigen for antibody function is the native or the recombinant TMV 30K movement protein or domains thereof.
4) Additionally to the bioassays listed in example 1 generated transgenic plants will be tested for broad spectrum resistance against different viral strains or viral genera by inoculation of transgenic plants with virions or infectious transcripts.

Anyone of skill in the art will recognise that these steps can be followed for any other viral pathogen by selecting antibodies or fragments thereof specific for the movement protein and any functional domain. Moreover, example 5 can be combined with examples 1-4 and 6-8 in any combination(s).

Generation of anti-30K movement protein specific antibodies by phage display

Movement protein (MP) - specific recombinant antibodies were generated by phage display using bacterially expressed 30K TMV movement protein fused to GST to

faciliate affinity purification (Pharmacia GST-System). Female Balb/c mice were immunized using a standard protocol with soluble GST-30K fusion proteins or domains thereof. The native structure of the GST and the fused 30K was tested by GST activity assay (Smith et al., *Gene* 67 (1988), 31-40) and RNA binding of the 30K (Vaquero, J. Gen. Virol. 78 (1997) 2095-2099). Antibody V_H- and V_L- regions were subsequently rescued from plasma cells of hyperimmunized mice and assembled to scFv antibodies using an SOE-PCR protocol (Mc Cafferty et al., Nature 348 (1990), 552-554). An extended set of PCR-primers for V_H and V_L family specific amplification was developed and used (Table 4). Using this protocol a library > 10^6 different scFv was generated for scFv presentation on the phage surface in fusion to the gene III M13-pilot protein. Using phage display a panel of 10 different movement protein specific antibodies could be isolated and characterised which all showed strong binding affinity to the bacterial expressed GST-30K fusion protein and not to the bacterially expressed GST-protein alone (Figure 20a). The binding domain of these 10 antibodies on the 30K were mapped by expression of 5 distinct 30K domains and ELISA analysis (Figure 20b). For evaluation of the biological effects of plant expressed anti 30K antibodies the 6 strongest binders were subsequently cloned into plant expression vectors and expressed and analysed by phenotypic evaluation and molecular analyses to evaluate their effects on viral infection with TMV. From these experiments two plant lines expressing the antibody fragments scFv 30-1 and scFv 30-2 (Figure 21) showed significant inhibition on TMV infection confirmed by a healthy phenotype of the plants, reduced amount and delayed accumulation of TMV coat protein compared to wild type *N. tabacum* Petite Havana SR1.

Construction of the anti-30K antibody expression cassette for expression in plants

To express the anti-30K antibodies in the cytosol of *N. tabacum* cv. Petite Havana SR1, the antibody fragments were cloned into the plant expression vector pSSH1 (Figure 19) (Voss et al., Molecular Breeding 1 (1995), 39-50). The cloning of the bispecific antibody fragment BISCA2429, which was used as parental construct for anti-30K antibody expression, including the TMV-derived Omega sequence, into the plant expression vector pSSH1 has been described (Fischer, et al., (1999) *Eur. J. Biochem.* 262, 810-816). For cloning of the resulting scFvs into this vector the primers used for establishing the phage display library contained the restriction enzyme sites *Nco* I and *Sal* I which faciliated the in-frame cloning into the

intermediate construct pUC18-BISCA2429 (5´-UTR of TMV omega sequence, scFv, His6 tag). For expression in plant cells, the *EcoR*I/*Xba*I fragment (Figure 19) of this intermediate construct was subcloned into the *EcoR*I and *Xba*I restriction sites of the plant expression vector pSS containing the enhanced 35S promoter (Kay et al., Science 236 (1987), 1299-1302) and the CaMV termination sequence (Figure 19). The final constructs contained an expression cassette starting with the 35 SS promoter followed by the 5´- UT of the TMV omega sequence, the 30K specific scFv, a His6 tag for affinity purification and the 3´- UTR of CaMV (Figure 19 and 21).

Generation and molecular characterization of transgenic plants

Upon completion of scFv analysis *in vitro,* tobacco plants were stably transformed for *in vivo* testing of scFv effects on TMV infection. Transgenic *N. tabacum* cv. Petite Havana SR1 were generated by leaf disc transformation using recombinant *A. tumefaciens* and transgenic T_0 plants were generated from transformed callus (Horsch et al., Science 227 (1985), 1229-1231). From the T_0 generation several plants were selected and homozygous T_1 and T_2 progenies selected for phenotypic and molecular evaluation of scFv-antibody mediated resistance.

To test if the anti 30K-specific scFv expression in transgenic plants had an biological effect on TMV replication and spread within the plant, a time course based TMV coat protein assay was established where the amount of viral coat protein was monitored in upper leaves after infection of transgenic and nontransgenic plants with TMV *vulgare*. Extraction of TMV coat protein from systemically infected tobacco leaves and subsequent analysis on SDS-PAGE (Laemmli, Nature 227 (1970), 680-685) was performed as described by Fischer *et al.* (Fischer et al., (1998) In: Cunningham C, Porter AJR (eds), Recombinant proteins in plants: Production and Isolation of Clinically useful compounds, pp. 45-68. Vol. 3. Humana press, Totowa, NJ). Monitoring the coat protein expression in systemically infected wild type and control tobacco leaves by western blotting revealed increasing amounts of the TMV coat protein. In case of plants expressing the 30K specific scFvs, accumulation of coat protein was significantly delayed and levels were significantly lower compared to wild type *N. tabacum* cv. Petite Havana SR1, and transgenic N. tabacum Petite Havana SR 1 expressing a non-related scFv, used as control (Figure 22). Although coat protein was detectable in all transgenic plants they developed no or only mild symptoms based on phenotypic evaluations (Table 3).

Bioassays to test viral resistance

To analyse the biological effects of the TMV-specific 30K scFvs on viral resistance, T_1 and T_2 progenies of plant lines expressing the scFv 30-1 and scFv 30-2 S (Figure 21) were inoculated with TMV. Seeds were collected from different T_0 plants and germinated on MSMO agar medium supplemented with 2 % (w/v) sucrose, 0.4 μg/ml thiamine, 0.4 μg/ml glycine, 0.1 μg/ml nicotine acid, 0.1 μg/ml pyridoxine and 75 μg/ml kanamycin. Germinating plants were transferred to soil and fully grown T_0 plants self fertilized for seed collection. Kanamycin-resistant homozygous T_1 and T_2 plants were used for inoculation with TMV-*vulgare* (40 ng/ml). Four to six week old plants were mechanically infected on two lower leaves using 200 μl of the TMV containing suspension. *N. tabacum* cv. Petite Havana SR1 wild type or transgenic plants producing the tumorspecific scFv T84.66 as a control were inoculated as described. Disease symptoms were monitored 7 to 14 days post inoculation (p.i.).

Upon infection of the two lower leaves with TMV, systemic spread of the virus was monitored by phenotypic evaluation of upper leaves after 7, 9, 11 and 14 days p.i. All control plants were systemically infected, and showed increasing amounts of viral coat protein. Transgenic T_1 and T_2 plants derived from different T_0-plant lines expressing scFv 30K-1 or scFv 30K-2 showed no visible or weak disease symptoms on the upper leaves (Table 3). In cases where the symptom development was visible this was consistent among plants derived from one plant line. In the case of the scFv 30K-2 one plant line was obtained which showed severe symptoms in the T_1 as well as in the T_2 generation. This may be due to positional effects of the transgene or silencing effect of the transgene in the T_1 and T_2 generation. Furthermore, SDS-PAA based analysis and subsequent western-blotting of TMV coat protein accumulation over 72 hrs p.i. in infected plants demonstrated that all of these plants accumulated virus particles in the upper leaves although to a lower degree (Figure 22). This lead to the conclusion that expression of movement protein specific scFv can prevent symptom onset and delay on CP accumulation upon TMV infection.

Other viral movement proteins

To broaden the application of antiviral antibodies in transgenic plants for engineering resistance the 3a movement protein of cucumber mosaic virus was expressed in *E. coli* as a GST-fusion (Vaquero et al., (1997) J. Gen. Virol. 78, 2095-2099). Specific

anti-3a antibodies were generated by phage display and hybridoma technology using bacterially expressed 3a CMV movement protein as already described for the 30K movement protein of TMV (Figure 19). The mAb 3a-2 was cloned from hybridoma cells (Krebber et al., (1997). J. Immunol. Methods 201, 35-55) and the resulting scFv 3a-2 characterized *in vitro* by ELISA technique (Figure 23a). Using epitope mapping by peptide display the epitope of the antibody could be mapped to a distinct region on the 3a movement protein.

Table 3:

Phenotypic evaluation of TMV-30K-specific scFv expressing transgenic *N. tabacum* plants in the T_1 and T_2 generation. Different plants were tested from the T_1 and the T_2 generation for phenotypically visible disease symptoms upon infection of two lower leaves with 40 ng/ml TMV. Disease symptoms were monitored at 7, 9, 11 and 14 days p.i. in the most upper leaves.

Construct	T_1-Plants		T_2- Plants	
	No. of infected plants	symptoms	No. of infected plants	symptoms
pSS-scFv-30-1	55	no symptoms after 11 to 14 days	22	weak symptoms after 14 days in all plants
pSS-scFv-30-2	124	weak or medium, rarely severe symptoms	65	no to weak, rarely severe symptoms

Table 4:

Primers used for amplifying murine V_H and V_L antibody domains for scFv generation. These primers were used for cloning of scFvs prior to phage display as well as for cloning scFvs from preexisting hybridoma cell lines ("hybridoma rescue"). The specificity of all primers according to the Kabat-database (Kabat: "Sequences of immunological interest", 1991) is listed, all primers contain a 5´- noncoding 10 bp overhang to faciliate restriction enzyme digestion and cloning. For cloning V_H-fragments restriction enzymes *Sfi* I, *Fse* I, for cloning V_L-fragments restriction enzymes *Asc* I/ *Not* I were used.

Name	Specificity according to Kabat (1991)	Overhang/ Enzyme Region	Annealing region
MPD VHF 1	Mu V_H IA Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	GAK GTR CAG CTT CAG GAG TCR GGA
MPD VHF 2	Mu V_H IB Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	CAG GTG MAG CTG AWG GAR TCT GG
MPD VHF 3	Mu V_H IIA Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	GAG GTC CAG CTR CAR CAR TCT GGA CC
MPD VHF 4	Mu V_H IIA Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	CAG GTW CAG CTS CAG CAG TCT G
MPD VHF 5	Mu V_H IIB Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	SAG GTC CAR CTG CAG SAR YCT GGR
MPD VHF 6	Mu V_H IIC Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	GAG GTT CAG CTG CAG CAG TCT GGG
MPD VHF 7	Mu V_H IIIA Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	GAR GTG AAG CTG GTG GAR TCT GGR
MPD VHF 8	Mu V_H IIIB Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	GAG GTG AAG STY MTC GAG TCT GGA
MPD VHF 9	Mu V_H IIIC Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	GAR GTG AAG CTK GAK GAG WCT GR
MPD VHF 10	Mu V_H IIID Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	GAV GTG MWG CTK GTG GAG TCT GGK
MPD VHF 11	Mu V_H IIID Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	GAG GTG CAR CTK GTT GAG TCT GGT G
MPD VHF 12	Mu V_H VA Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	SAG GTY CAG CTK CAG CAG TCT GGA
MPD VHF 13	Mu V_H 1 Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	CAG ATC CAG TTG GTG CAG TCT GGA
MPD VHF 14	Mu V_H 2 Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	CAG GTS CAC STG RWG SAG TCT GGG
MPD VHF 15	Mu V_H 3 Front	CAG GTS CAC STG RWG SAG TCT GGG	CAG GTT ACT CTR AAA GWG TST GGC C
MPD VHF 16	Mu V_H 4 Front	C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC	GAT GTG AAC TTG GAA GTG TCT GG
MPD VLF1	Mu kappa V_L I Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAC ATT GTG MTG WCH CAG TCT CCA
MPD VLF2	Mu kappa V_L I Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAC ATT CAG ATG ATT CAG TCT CC
MPD VLF3	Mu kappa V_L I Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAC ATT GTT CTC WHC CAG TCT CC
MPD VLF4	Mu kappa V_L I Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAC ATT GTG MTG WCH CAG TCT CAA
MPD VLF5	Mu kappa V_L II Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAT RTT KTG ATG ACC CAR RCK GCA
MPD VLF6	Mu kappa V_L II Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAT RTT KTG ATG ACC CAR RCK CCA
MPD VLF7	Mu kappa V_L II Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAC ATT GTG ATG ACC CAR BHT G
MPD VLF8	Mu kappa V_L II Front	CAT GCC ATG ACT CGC GGC	GAT ATT KTG ATG ACC CAR AYT

Name	Specificity according to Kabat (1991)	Overhang/ Enzyme Region	Annealing region
		GCG CCT	CC
MPD VLF9	Mu kappa V_L III Front	CAT GCC ATG ACT CGC GGC GCG CCT	RAM ATT GTG MTG ACC CAA TYT CCW
MPD VLF10	Mu kappa V_L IV Front	CAT GCC ATG ACT CGC GGC GCG CCT	SAA AWT GTK CTS ACC CAG TCT CCA
MPD VLF11	Mu kappa V_L V/VI Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAY ATY CAG ATG ACM CAG WCT AC
MPD VLF12	Mu kappa V_L V/VI Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAY ATY CAG ATG ACH CAG WCT CC
MPD VLF13	Mu kappa V_L V/VI Front	CAT GCC ATG ACT CGC GGC GCG CCT	GAC ATT GTG ATG ACT CAG GCT AC
MPD VLF14	Mu lambda V_L 1 Front	CAT GCC ATG ACT CGC GGC GCG CCT	CAR SYT GTK STS ACT CAG KAA T
MPD VLF15	Mu lambda V_L 1 Front	CAT GCC ATG ACT CGC GGC GCG CCT	CAR SYT GTK STS ACT CAG KCA T
MPD VHB1	Mu V_H J_H 1 Back	CTA GTG GTA CTC CAC GGC CGG CCC CTG	MRG AGA CDG TGA SMG TRG TC
MPD VHB2	Mu V_H J_H 2 Back	CTA GTG GTA CTC CAC GGC CGG CCC CTG	MRG AGA CDG TGA SRG TRG TG
MPD VHB3	Mu V_H J_H 3 Back	CTA GTG GTA CTC CAC GGC CGG CCC CTG	MRG AGA CDG TGA SCA GRG TC
MPD VHB4	Mu V_H J_H 4 Back	CTA GTG GTA CTC CAC GGC CGG CCC CTG	MRG AGA CDG TGA STG AGG TT
MPD VHB5	Mu V_H J_H 4 Back	CTA GTG GTA CTC CAC GGC CGG CCC CTG	MRG AGA CDG TGA STG ARA TT
MPD VLB1	Mu kappa V_L I/II/IV back	CT AGT GGT ACT CCA CGC GGC CGC GTC GAC	AGC MCG TTT CAG YTC CAR YTT
MPD VLB2	Mu kappa V_L I/II/IV back	CT AGT GGT ACT CCA CGC GGC CGC GTC GAC	AGC MCG TTT KAT YTC CAR YTT
MPD VLB3	Mu kappa V_L IV back	CT AGT GGT ACT CCA CGC GGC CGC GTC GAC	AGC MCG TTT BAK YTC TAT CTT TGT
MPD VLB4	Mu kappa V_L I/II/V back	CT AGT GGT ACT CCA CGC GGC CGC GTC GAC	AGC MCG AGC MCG TTT TAT TTC CAA MKT
MPD VLB5	Mu lambda V_L back	CT AGT GGT ACT CCA CGC GGC CGC GTC GAC	CTG RCC TAG GAC AGT SAS YTT GGT

Example 6: Enhanced resistance by the expression of antibodies against the Tobacco mosaic virus replicase

Expression of antibodies against the TMV 54K/TMV 183K replicase subunits in transgenic tobacco

The steps 1) to 16) of example 1 and/or the steps 1) to 2) of example 2 are repeated with the following adaptations:

1) Specific antibodies recognising the TMV 54K/183K replicase are raised by hybridoma and phage display or ribosome technology by using recombinant TMV 54K / TMV 183K proteins as the antigen and cloned to engineer single chain antibody fragments or any recombinant form thereof including bispecific scFvs.
2) These antibodies are expressed in the cytosol or targeted to cytoplasmic face of intracellular membranes, where the virus replication complexes are formed, by using a C-terminal sequence as described in example 2. The recombinant antibody may cause the desired biological effect without a fusion to a toxin.
3) For ELISA and surface plasmon resonance the test antigen for antibody function is the native or the recombinant TMV 54K and 183K replicase proteins or domains thereof.
4) Additionally to the bioassays listed in example 1, generated transgenic plants will be tested for broad spectrum resistance against different viral strains or viral genera by inoculation of transgenic plants with virions or infectious transcripts.

Anyone of skill in the art will recognise that these steps can be followed for any other viral pathogen by selecting antibodies or fragments thereof specific for the movement protein and any functional domain. Moreover, example 6 can be combined with examples 1-5 and 7-8 in any combination(s).

Anti-replicase specific scFv (scFv 54K)

An alternative method to prevent viral infection is based on the intracellular expression of antibodies such as replicase specific scFvs which can interact with the viral replicase to interfere or inhibit viral proliferation in infected cells. As described in example 5 for the 30K movement protein the "54K protein" of TMV, was expressed in *E. coli* and antibodies were generated using standard hybridoma technologies available to anyone skilled in the art. The 54K protein can be considered as an integrative component of the 183K protein, the major replicase protein of TMV, since it is expressed from its own subgenomic RNA and promoter but it shares the same

reading frame of the last 1400 bases of the 183K protein. Herein conserved regions such as the GDD-Motif are encoded which can be found in all plant viral replicases. Antibody V_H and V_L regions were cloned from hybridomas using the same set of primers described in example 5 and assembled into scFv antibodies using standard cloning procedures (Krebber et al. (1997), J. Immunol. Methods 201, 35-55). The activity of the resulting scFv antibody scFv 54-1 (Figure 23a) was monitored by western blot detection and ELISA using the bacterial expressed GST-54K. The epitope of the scFv 54-1 was determined by peptide display (Figure 25b) and could be mapped to a distinct region on the 54K/ 183K gene of TMV.

Plant virus minimal proteins

Alternative viral proteins for engineering viral resistance are the "plant viral minimal proteins" 1min, 2min and 3min as decribed for PLRV. One characteristic of the minimalprotein 3min is its ability to bind to nucleic acids wherby preferentially single stranded RNA is bound (Figure 24) (Prüfer et al., (1992) EMBO Journal 11, 1111-1117). 3min-specific antibodies were generated by hybridoma technology using bacterial expressed GST-3 min as antigen and the antibody scFv 3min was cloned from hybridoma cells (Figure 23c). Since the 3min protein of PLRV is described as a nucleic acid binding protein the native structure of the bacterial expressed GST-3min could be confirmed by nucleic acid binding assays using GST 3min and *in vitro* transcripts of viral RNA and DNA. Using the antibody scFv 3 min the RNA/DNA binding activity of the 3min protein could be completely blocked *in vitro*. Using epitope mapping by peptide display the epitope of the antibody could be mapped to a distinct region on the 3min minimal protein wherby the epitope identified by peptide display overlapped with the GST cloning region of the GST-3min construct (Figure 25c).

Epitopemapping of antiviral scFvs (scFv 29, 3min and 54 K-1)

For elucidation of viral epitopes recognised by the developed recombinant antibodies two peptide display libraries were used for epitope mapping of monoclonal antibodies (Cortese et al. (1995), Curr. Opin. Biotechnol. 6, 73-80). Both libraries express 9mer random peptides at the N-terminus of filamentous phage pVIII protein. One library displays the peptides in linear form, the other library displays the peptides flanked by two Cysteine-residues which form a disulfide-bridge, constraining the peptide to a

loop structure.

To identify the epitopes of scFv 29, scFv 54-1, and scFv 3min immunotubes were coated using 20µg affinity purified antigen using a standard procedure available to anyone skilled in the art (Cortese et al., (1995) Curr. Opin. Biotechnol. 6, 73-80). For the first round of panning, 5 x 10^{12} phages in 1ml PBS were incubated with the immobilized antigen (16 h, 4°C). After extensive washing (15 times PBST and 5 times PBS), bound phages were eluted with 1ml Glycine-HCl pH 2.2, 0.1% (w/v) BSA (10min, 20°C), neutralized with 60 µl 2M Tris and used for infection of *E. coli*. The titer of eluted phages was determined by plating 100 µl of the infected bacteria on 2 x TY-Amp-plates and counting the colony forming units. Enrichment factors were calculated upon comparison to a control panning using BSA as antigen. Monoclonal phages from the third round of panning were tested for reactivity to their antigens by phage-ELISA.

Positively identified phages from phage ELISA, using both phage libraries individually on all scFvs, were subsequentely sequenced. Obtained sequences were aligned and the resulting consensus sequence was determined. In all three cases (scFv 29, scFv 54-1 and scFv 3min) a consensus sequence could be determined (Figure 25). The resulting consensus sequence could also be mapped back to the parental sequence of the antigen the antibodies were generated against. Since the 9mer random peptide library presents preferentially linear peptides it is considered that all three epitopes of the scFvs represent a linear motif on the antigen. The epitope of scFv29, scFv54-1 and scFv3min were determined by peptide display and a consensus sequence was mapped for each scFv (Figure 25). For the scFv29, the consensus sequence was mapped to a distinct region of the coat protein (Figure 25a) and the scFv54-1 consensus sequence was mapped to a distinct region of the TMV 54K protein (Figure 25b). For the scFv3min the consensus sequence contained part of the GST and 3min proteins (Figure 25c) at the point where the 3min was fused to the GST. ScFv3min can be considered useful for engineering virus resistance as it inhibits the nucleic acid binding activity of the 3min protein (Figure 24).

Example 7: *In vivo* assembly of a molecular pathogenicide

In vivo assembly of a molecular pathogenicide consisting of a TMV specific antibody labelled with an epitope specific single chain antibody and an epitope tag labelled toxin

The following steps are taken:

1) Antibodies are generated against intact TMV virions and monoclonals are generated by hybridoma technology.

2) Hybridoma cell lines are cloned and cDNA sequences encoding the antibody variable regions are cloned to generate a single chain antibody or any recombinant version thereof binding to the TMV virions (scFv24).

3) The single chain antibody binding to the intact virions (scFv24) is fused to a cloned cDNA from the single chain antibody (scFv-epitag29), which binds to a specific amino acid epitope (epitag29), using a flexible linker such as the linker peptide of *Trichoderma reesi* cellobiohydrolase I (CBHI) to generate a recombinant protein which recognises the pathogen, TMV, and the epitope tag. The scFv-epitag29 has been previously generated (by conventional hybridoma technology and then cloned as an scFv) and the specific epitope identified by phage peptide display. Any other high affinity antibody recognising an identified peptide epitope would be suitable as one half of the binding pair with its corresponding epitope as the other partner.

4) The recombinant gene from step 3 is inserted in a microbial or eukaryotic expression vector.

5) The binding specificity and function (i.e. specificity and affinity) of the recombinant protein from step 3 is checked after expression in a heterologous host, such as in the periplasm of *E.coli*.

6) A signal sequence is added to the N-terminus of the recombinant bispecific scFv construct from step 3, to permit delivery of the protein to the ER and secretion to the apoplast upon expression in plants. A 5' untranslated and a 3' untranslated region and a detection tag sequence (i.e. c-myc) will be introduced by recombinant DNA technology, if necessary.

7) The chimeric gene from step 6 is inserted into a plant expression vector, e.g. pSS (Voss et al., 1995). Suitable plant expression vectors include suitable promoter, enhancer, terminator and selection marker sequences. In case of markerless and vectorless gene transfer selection marker sequences can be

omitted.

8) The cDNA encoding a RIP (ribosome inactivating protein) fused via a suitable linker to the epitag-29 epitope tag (either at the N- or C-terminus), which is specifically recognised by scFv-epitag29, is prepared in parallel to generate a second independent expression construct encoding a tagged RIP gene.

9) The tagged RIP gene is inserted in a microbial or eukaryotic expression vector and the functionality of the RIP-epitope fusion is checked upon expression in a heterologous host.

10) A second, independent plant expression vector, such as pSS, containing the recombinant tagged RIP gene with an N-terminal signal peptide will be prepared, as described in step 6 and step 7. The tagged RIP sequence can then be integrated either in tandem array on the same plasmid as the fusion protein from step 3 or integrated in a second independent plasmid. Note that the sequences remain discrete even if they are in tandem array.

11) Both plant expression constructs listed in steps 6 and 10 are transformed into two independent plant lines or they are co-transformed into the same plant genome, or if the antibody fusion protein from step 3 and the tagged toxin from step 10 are integrated in tandem array that construct is transformed into the same plants.

12) Regenerated plants are screened using the selection marker for integration of the fusion gene in the independent plant lines or the co-transformed lines from step 11.

13) Transgenic plants that express only one of either the antibody fusion protein from step 3 or epitope tagged RIP from step 10 are then sexually crossed to give offspring which will produce both proteins. Plants producing both proteins, whether from this or earlier steps, will produce assembled protein complexes were the two binding partners, the epitope specific antibody (scFv-epitag29) and the epitope bind and permit assembly of a molecular pathogenicide protein complex.

14) Expression of the bispecific scFv fusion protein and/or the tagged RIP as well as the *in vivo* assembled molecular pathogenicide is monitored by western blotting cell extracts, ELISA or surface plasmon resonance analysis. Activity of the bispecific scFv is checked by ELISA using intact TMV virions as the antigen.

15) Activity of the assembled molecular pathogenicide is checked by ELISA and cytotoxicity assays.

16) Localisation of the fusion protein is checked by indirect immuno-fluorescence, confocal microscopy or immuno-electron microscopy and western blotting or ELISA or surface plasmon resonanace analysis of the intercellular washing fluid.

17) The biological activity of the *in vivo* assembled molecular pathogenicide against TMV is assayed by bioassays on the generated transgenic plants using virions or infectious transcripts.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised and cloned against structural and non structural proteins of any pathogen. Instead of RIPs, similar toxins with cell killing activity or interference in pathogenicity (RNAase, DNase etc.) can be used. Assembly of molecular pathogenicides can be achieved by using any epitope tag and a given epitope specific antibody with a suitable and stable molecular interaction *in vivo,* or any other pair of proteins that bind to each other. Moreover, example 7 can be combined with examples 1-6 and 8 in any combination(s).

Constructs for analysing assembly of fusion partners

To analyse assembly of a molecular pathogenicide consisting of a TMV specific antibody labelled with an epitope specific single chain antibody and an epitope tag labelled toxin different constructs were generated and assembly was studied by immunoblot and ELISA.

TMV specific antibody labelled with an epitope specific single chain antibody (biscFv2429): The single chain antibody binding to the intact virions (scFv24) was fused to a cloned cDNA from the single chain antibody scFv-epitag29, which binds to a specific amino acid epitope (epitag29), using the flexible linker peptide of *Trichoderma reesi* cellobiohydrolase I (CBHI) to generate a recombinant protein which recognises the pathogen, TMV, and the epitope tag. The scFv-epitag29 has been previously generated (by convential hybridoma technology and then cloned as an scFv) as described for the scFv24 (Schillberg et al., *Transgenic Research* 8, 255-263 (1999)) and the specific epitope identified by phage peptide display. The mouse

N-terminal light chain signal peptide from the original antibody (mAb24) used to generate scFv24 was used to target biscFv2429 to the secretory pathway. For expression in plant cells, the 5' UT from the chalcone synthase was introduced and the cassette was inserted between the enhanced 35S promoter and the CaMV termination sequence in the pSS expression vector (Figure 26) (Fischer et al., *Eur. J. Biochem.* 262, 810-816 (1999)).

Epitope tag labelled toxin (PE280-tag29 and PE400-tag29): Two constructs were generated containing parts of the *Pseudomonas* exotoxin (PE) and the C-terminal epitag-29 fused via a Gly_4Ser linker. The construct PE280-tag29 (Figure 26B) contains the sequence from amino acid (aa) 280 to aa 609, which comprises domain II and domain III from PE. The construct PE400-tag29 (Figure 26C) contains PE domain III from aa 400 to aa 609. PE domain III mediates the ADP-ribosylation of elongation factor 2, which arrests the protein synthesis and causes cell death. The epitag-29, which is specifically recognised by scFv-epitag29, was fused C-terminal to both constructs via Gly_4Ser linker. The mouse N-terminal light chain signal peptide from the murine monoclonal antibody mAb24 was used to target PE280-tag29 and PE400-tag29 to the secretory pathway.

The PE280 and PE400 were PCR amplified from the plasmid PE38 using the following primers: for PE280: PE280-for 5'- GCG GAA TTC GAC GTC GCC ATG GGC TGG GAA CAA CTG GAG CAG -3' (SEQ ID NO:157) and PE-back 5'- GCG AAG CTT GTC GAC CGG CGG TTT GCC GGG CTG GCT G - 3' (SEQ ID NO:158); for PE400: PE400-for 5'- GCG GAA TTC GAC GTC GCC ATG GCC TTC CTC GGC GAC GGC GGC GAC - 3' (SEQ ID NO:159) and PE-back 5'- GCG AAG CTT GTC GAC CGG CGG TTT GCC GGG CTG GCT G - 3' (SEQ ID NO:160). The primers contained restriction sites *Eco*RI, *Aat*II and *Nco*I (PE280-for and PE400-for) and and *Hind*III (PE-back) for cloning. The PCR fragments were subcloned via *Eco*RI and *Hind*III into pUC18 resulting in the constructs PE280- and PE400-intermediate and the sequence was verified by sequencing. The *Nco*I/*Sal*I fragment from both constructs (PE280- and PE400-intermediate) was subcloned into a pUC18 derivate containing the chalcone synthase 5' untranslated region, the codon optimized leader signal from mAb24 light chain and the C-terminal epitope tag29 resulting in the constructs PE280-tag29 and PE400-tag29. For expression in plant cells, the *EcoR*I/*Xba*I fragments (Figure 26) of PE280-tag29 and PE400-tag29 were subcloned

into the *EcoR*I and *Xba*I restriction sites of the plant expression vector pSS (Voss et al., Molecular Breeding 1: 39-50 (1995)) containing the enhanced 35S promoter (Kay et al., Science 236: 1299-1302 (1987)) and the CaMV termination sequence (Figure 26B and C).

Control construct, epitope tag labelled GST (GST-tag29): To analyse the assembly of the epitag29 and the corresponding antibody, the control construct GST-tag29 was generated by introducing the epitag-29 sequence into the pGEX-5X-3 vector (Pharmacia) at the C-terminus of GST.

Analysis of GST-tag29

The epitag-29 is specifically recognised by scFv-epitag29 and the corresponding recombinant full-size antibody mAb29 or its recombinant version rAb29. To analyse the binding specificity of rAb29 to epitag-29, affinity purified, bacterial expressed GST-tag29 was diluted either in PBS or in protein extract of a wild type *N. tabacum* cv. Petite Havana SR1 plant and serial dilutions were seperated on a SDS-PAA gel and blotted onto a nitrocellulose membrane.

The recombinant plasmid GST-tag29 was transformed into *E. coli* BL21 (DE3) (Stratagene, La Jolla. CA, USA) and the fusion protein was expressed by inducing a log phase culture with 0.2-1.0 mM IPTG for 1-3 h at 30°C. GST-tag29 fusion protein was affinity purified on glutathione agarose by batch purification according to the manufacturer's instructions (Pharmacia). Serial dilutions of GST-tag29 were resolved by SDS-PAGE and blotted onto Hybond^{TM}-C nitrocellulose membranes (Amersham, Braunschweig, Germany). The membranes were blocked overnight with 2% non-fat skim milk in PBS (MPBS) at 4°C followed by incubation with rAb29 (Schillberg, et al., *Transgenic Research* 8, 255-263 (1999)) at room temperature for 2 h. Bound antibodies were detected using goat anti-mouse IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA, USA) and the substrates ρ-nitrobluetetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

As shown in Figure 27A, 80 ng GST-tag29 in PBS was detectable using rAb29. Although in tobacco extract the detection limit decreased to 200 ng GST-tag29 (Figure 27B), this result indicates that plant derived proteins did not prevent binding of rAb29 to the epitag-29.

The *in vivo* assembly in a plant cell was simulated by ELISA, in which the scFv-epitag29 bound to epitop-29. As shown in Figure 28, a significant OD reactivity was detectable, indicating that binding of the two partners took place in plant extracts.

Transient expression in tobacco leaves

To analyze the protein level of the recombinant PE280-tag29, *N. tabacum* cv. Petite Havana SR1 leaves were transiently transformed with recombinant *A. tumefaciens* and accumulation of PE280-tag29 was analyzed by immunoblot.

Plant expression construct PE280-tag29 was transferred into *A. tumefaciens* GV3101 by N_2 transformation (Höfgen and Willmitzer, Nucleic Acids Res 16 (1988) 9877). Transient transformation of *N. tabacum* cv. Petite Havana SR1 was performed as described (Kapila et al., Plant Science 122 (1996) 101-108). To extract total soluble proteins, Tobacco leaves were frozen and ground in liquid nitrogen and scFv-fusion protein level was analysed by Western blot (Fischer et al., in: C. Cunningham, A.J.R. Porter, (Eds.), Methods in biotechnology Vol. 3: Recombinant proteins in plants: Production and Isolation of Clinically useful compounds. Humana Press, Totowa, NJ (1998)).

A protein of the expected size (37.1 kDa) was not detectable in plant extracts (Figure 29). However a degradation product was detectable, indicating that the recombinant PE280-tag29 protein accumulated in tobacco leaves. The plant leaves showed a healthy phenotype, indicating that PE280-tag29 was most likely secreted to the apoplast and therefore not toxic to the plant cell.

Characterization of transgenic plants

N. tabucum cv. Petite Havana SR1 was transformed with the construct PE280-tag29 using recombinant agrobacteria. Transgenic *N. tabacum* cv. Petite Havana SR1 were generated by the leaf disc transformation with recombinant *A. tumefaciens* and transgenic T_0 plants were generated from transformed callus (Horsch et al., Science 227: 1229-1231 (1985)). Regeneration of transgenic plants is in progress.

In addition, transgenic *N. tabucum* cv. Petite Havana SR1 plants accumulating biscFv2429 in the apoplast will be retransformed with the plant expression construct PE280-tag29 to analyse *in vivo* assembly and biological effects of the molecular pathogenicide.

Conclusions

The Molecular Pathogenicide will be assembled in the ER via the scFv-epitag29 and epitag29. The presented experiments show that proteins can be assembled using scFv-epitag29 and epitag29 in plant extracts. Upon assembly the Molecular Pathogenicide will be secreted to the apoplast. During TMV infection the fusion protein will bind to the virus particle via the scFv24 part and TMV virions that enter the cell will carry bound scFv24-toxin fusion. PE400 mediates the ADP-ribosylation of elongation factor 2, which arrests protein synthesis and causes cell death. PE is a very effective toxin as only a few molecules are required to kill the infected cell, thus preventing virus replication and spread, leading to highly resistant plants.

Example 8: *In vivo* proteolysis

The steps 1) to 16) of example 1 and steps 1) to 2) example 2 are repeated with the following adaptations.

1) A protease cleavage sequence which is processed by a plant and/or a pathogen protease *in vivo* is added either between the recombinant scFv construct and the C-terminal membrane localisation sequence, using a suitable linker, or between an N-terminal toxin and a C-terminal membrane anchored recombinant antibody or *vice versa*.
2) The chimeric gene is inserted into a plant expression vector e.g. pSS (Voss et al., 1995).
3) Suitable protease cleavage sequences include a selected sequence from a random linker library (Doskeland, Biochem. J. 313 (1996), 409-414) that had been selected by *in vitro* proteolysis and any known protease site that is unique to the fusion protein and does not destroy the molecular viricide and its activity *in vivo*. As an example, the c-myc tag or the CBHI linker is sensitive to plant proteases.

Anyone of skill in the art will recognise that these steps can be followed for any other pathogen by selecting antibodies or fragments thereof specific for the target pathogen. For example, antibodies can be raised and cloned against structural and non structural proteins of any pathogen. In addition, toxins can be cloned C- or N-terminal of the protease cleavage sequence. Toxins include all proteins and peptides that have a detrimental or toxic effect on a pathogen during its life cycle and/or an effect on the pathogen during plant infection or pathogen replication, spread or

transmission. This includes toxins that specifically kill an infected host cell and so limit the spread and development of a disease. Moreover, example 8 can be combined with examples 1-7 in any combination(s).

Construction of the scFv24 fusion expression cassettes

To integrate the TMV-specific scFv24 into the plant cell membrane, the antibody fragment was fused to an N-terminal mammalian signal peptide and C-terminal receptor transmembrane domain. The mouse N-terminal light chain signal peptide from the parental antibody (mAb24) used to generate scFv24 was used to target fusion proteins to the secretory pathway. We selected the transmembrane domain sequences of the human platelet derived growth factor receptor (PDGFR) for fusion with the C-terminus of scFv24, for heterologous targeting of the scFv24 antibody to the plasma membrane. To ensure proper folding of the expressed and subsequent cleaved single chain antibody fragment, the construct contained the c-*myc* sequence (pscFv24-PDGFR) as a linker and cleavage sequence between the scFv24 fragment and the membrane anchor (Figure 30).

To construct pscFv24-PDGFR, the cDNA encoding the c-*myc* epitope followed by the human platelet-derived growth factor receptor (PDGFR) transmembrane domain (18) was excised from the pHOOK-1 vector (Invitrogen, Leek, Netherlands) and ligated into the *Sal*I and *Xba*I restriction sites of the pscFv24-TcRβ plasmid (Example 1) to generate the pscFv24-PDGFR fusion construct (Figure 30).

Expression of the scFv24 fusion protein in *N. tabacum* cv. BY-2 cell suspensions

To analyze the expression level of the recombinant scFv-fusion proteins, the suspension cell line *N. tabacum* cv. BY-2 was stably transformed with recombinant *A. tumefaciens* and functional expression of the scFv24 domain of the fusion protein was analyzed by ELISA using anti-mAb24 antisera.

The vector construct pscFv24-PDGFR was transferred into *A. tumefaciens* GV3101 by liquid N_2 transformation (Höfgen and Willmitzer, Nucleic Acids Res. 16: 9877 (1988)). *N. tabacum* L. cv. bright yellow 2 (BY-2) cells were maintained in Murashige and Skoog basal salt with minimal organics (MSMO+: MSMO (Sigma, Deisenhofen, Germany) plus 200 mg/ml KH_2PO_4, 0.6 µg/ml thiamine, 3 % sucrose and 0.2 µg/ml 2,4-D, pH 5.8) at 24°C in the dark on an orbital shaker. Cells were subcultured every week with a 5 % inoculum. Three days after subculture, plant cells were transformed

by co-cultivation with recombinant *A. tumefaciens*, as described (An, Plant Physiol. 79: 568-570 (1985)). Selection of kanamycin-resistant transformants was performed on MSMO+ agar medium supplemented with 75 µg/ml kanamycin and 100 µg/ml claforan.

For extraction of total soluble proteins from transgenic BY-2 suspension culture, cells from 1 ml culture were collected by centrifugation at 4000 x *g* for 5 min at 4°C. The cell pellet was resuspended in 1 ml protein extraction buffer (200 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02 % (w/v) sodium-azide and 0.1 % (v/v) Tween 20) and cells were disrupted by sonication at 60 watt for 1 min using a sonicator probe (B. Braun, Melsungen, Germany) at 4°C. Cell debris was removed by centrifugation at 14000 x *g* for 10 min at 4°C. The clear supernatant containing soluble protein was used for further analysis.

For ELISA and western blotting, anti-mAb24 antisera (Zimmermann et al., Molecular Breeding 4: 369-379 (1998)) or the anti-c-*myc* monoclonal antibody 9E10 (Evan et al., Mol. Cell. Biol. 5: 3610-3616 (1985)) were used as a primary antibody in combination with a 1:5000 dilution of goat anti-rabbit or goat anti-mouse alkaline phosphatase conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA). Protein concentrations were determined with the Bio-Rad Protein Assay using Bovine Serum Albumin (BSA) as standard.

Analysis of stably transformed *N. tabacum* BY-2 cells revealed that scFv24-PDGFR was present in both cell extracts and culture supernatant (Figure 31). Transgenic cell suspension lines showed similar expression levels for both recombinant proteins, but 44-88 % (mean value = 69 %, n = 5) of scFv24-PDGFR was secreted into the culture supernatant. This indicated that the scFv24-PDGFR is released by proteolysis from the plasma membrane.

Characterization of transgenic plants

We then tested whether the heterologous mammalian transmembrane domain PDGFR fused to scFv24 would target the single chain antibody to the plasma membrane in stably transformed tobacco plants. Transgenic *N. tabacum* cv. Petite Havana SR1 were generated by the leaf disc transformation with recombinant *A. tumefaciens* and transgenic T_0 plants were generated from transformed callus (Horsch et al., Science 227: 1229-1231 (1985)). Extraction of total soluble proteins from tobacco leaves and subsequent analysis of scFv24 by ELISA were performed

as described by Fischer *et al.* (Fischer et al., in: Cunningham C, Porter AJR (eds), Recombinant proteins in plants: Production and Isolation of Clinically useful compounds, pp. 45-68. Vol. 3. Humana press, Totowa, NJ (1998)). For ELISA and western blotting, anti-mAb24 antisera (Zimmermann et al., Molecular Breeding 4: 369-379 (1998)) was used as a primary antibody in combination with a 1:5000 dilution of goat anti-rabbit alkaline phosphatase conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA). Protein concentrations were determined with the Bio-Rad Protein Assay using Bovine Serum Albumin (BSA) as a standard.

Expression levels of scFv24-PDGFR were much higher in transgenic *N. tabacum* cv. Petite Havana SR1 plants than in suspension cultures (Table 5). The maximum level of detergent extracted scFv24-PDGFR was 13 fold higher (388 ng/g leaf tissue) than that obtained in transgenic suspension cultures (Figure 31).

In *N. tabacum* cv. BY-2 suspension cells producing the scFv24-PDGFR fusion protein, scFv24 was detectable in the culture supernatant. To determine if scFv24 fragments were secreted into the extracellular space of intact plants, intercellular washing fluid from leaves of transgenic T_1 tobacco plants was analyzed by ELISA. For detection of scFv24 fusion proteins in intercellular washing fluids, leaves of *N. tabacum* cv. Petite Havana SR1 were prepared as described by Fischer *et al.* (Fischer et al., in: Cunningham C, Porter AJR (eds), Recombinant proteins in plants: Production and Isolation of Clinically useful compounds, pp. 45-68. Vol. 3. Humana press, Totowa, NJ (1998)). Total protein extracts from washing fluids were concentrated by ultrafiltration (Microcon 10, Amicon, Witten, Germany) and analyzed by 12% SDS-PAGE (Laemmli, Nature 227: 680-685 (1970)) followed by western blot. scFv24 was present in the intercellular washing fluid of seven progenies of a plant line ($P9_{SR1}$) producing scFv24-PDGFR and the level of secreted scFv24 did not correlate to levels of protein expression in intact leaves. In general, T_1 plants used for IWF analysis showed scFv24 expression levels of 1080-1540 ng/g leaf tissue. Therefore, the protein is cleaved by a host protease.

Western blot analysis, using an anti-mAb24 antisera, revealed that a single 28kDa scFv24 polypeptide was detected in the intercellular washing fluid of T_1 progenies from scFv24-PDGFR transgenics ($P9_{SR1}$). This corresponded to a fusion protein cleavage product since the predicted molecular weight of scFv24-PDGFR was 35KDa. However, both the full length (35KDa) and cleaved (28KDa) fragments of

scFv24 were present in total soluble cell protein extracts at a 1:1 ratio of full-length product to the fragment (Figure 32A). Western blot analysis of total soluble protein extracts with an anti c-*myc* antibody only detected the intact fusion protein scFv24-PDGFR (Figure 32B). This indicates that the scFv24 fragment in the apoplast was cleaved off the membrane near or within the c-*myc* epitope tag.

Bioassays of viral resistance

To analyze the biological effects of the membrane anchored anti-viral TMV-specific antibody on viral resistance, T_1 progenies of plant line expressing the scFv24-PDGFR fusion protein ($P9_{SR1}$) were inoculated with TMV.

Seeds were collected from antibody-producing T_0 plants and germinated on MSMO agar medium supplemented with 2 % (w/v) sucrose, 0.4 μg/ml thiamine, 0.4 μg/ml glycine, 0.1 μg/ml nicotine acid, 0.1 μg/ml pyridoxine and 75 μg/ml kanamycin. Kanamycin-resistant T_1 plants were used for inoculation with TMV-*v* (1 μg/ml) as previously described (Dietzgen et al., Arch. Virol. 87: 73-86 (1986)). Wild type *N. tabacum* cv. Petite Havana SR1 plants were used as a control. Disease symptoms were monitored 6 to 20 days post inoculation (p.i.) and for resistant plants up to 180 days p.i..

Lower leaves were infected with TMV and systemic spread of the virus was followed by analyzing upper leaves 6-20 days later. All non-transgenic tobacco control plants were systemically infected, but 19 % (out of 68 analyzed) of scFv24-PDGFR transgenic plants had no visible disease symptoms on the upper leaves (Table 6). Furthermore, ELISA analysis demonstrated that some of these plants accumulated virus particles in the upper leaves indicating that though systemic viral spread occurred, no symptoms were developed. Strikingly, in 13 % of scFv24-PDGFR transgenic plants no virus was found in the upper leaves up to 90 days post inoculation. Virus could be detected at inoculation sites in the lower leaves by ELISA demonstrating that these plants had been efficiently inoculated with TMV. Antibody-fusion protein expression levels correlated with expression of TMV resistance (Table 6). Higher levels of scFv24 fusion protein expression led to an increased fraction of virus resistant plants.

Conclusions

It could be shown that the linker region between the scFv24 and the PDGFR transmembrane domain is sensitive to plant proteases, the scFv24 is cleaved off *in vivo* and secreted to the apoplast in transgenic plants. scFv24 retains its function post cleavage and creates a virus resistant phenotype.

Table 5:

Levels of functional scFv24 fusion protein in the T_0 generation of transgenic *N. tabacum* cv. Petite Havanna SR1.

Total soluble plant protein was isolated from leaves of transgenic plants producing scFv24-PDGFR. scFv24-fusion protein expression was quantified by TMV-specific ELISA using anti-mAb24 antisera and expressed as ng scFv24 per g leaf tissue.

Construct	Number of transgenic plants	Number of plants expressing functional scFv24	Range of expression (ng/g leaf tissue)	Average expression (ng/g leaf tissue)
pscFv24-PDGFR	12	9	3 - 388	114

Table 6:

Virus infection assay of trangenic plants expressing membrane anchored scFv24.

1 µg/ml TMV-*v* was applied onto a lower leaf of non-transgenic *N. tabacum* cv. Petite Havana SR1 and transgenic T_1 progenies from plant line $P9_{SR1}$ producing scFv24-PDGFR. scFv24-fusion protein levels were determined by ELISA using an anti-mAb24 antisera 14 days p.i. and used for group formation (low, average and high producers). [a] = upper leaves showed no visible disease symptoms; [b] = based on TMV-ELISA; [c] = level of resistance of all low, average and high producers, numbers in brackets include all plants without visible disease symptoms.

Plant lines	Tested plants	ng scFv24 per g leaf tissue	Healthy phenotype[a]	Resistant plants[b]	Level of resistance (%)[c]
N. tabacum cv.					
Petite Havana SR1	62	-	0	0	0
$P9_{SR1}$, low producer	34	10-500	1	0	
$P9_{SR1}$, average producer	23	501-2000	6	4	13 (19)
$P9_{SR1}$, high producer	11	2001-4200	6	5	

References

Anderson, J.M., P. Palukaitis, and M. Zaitlin. 1992. A defective replicase gene induces resistance to cucumber mosaic virus in transgenic sugarbeet plants. Proc. Natl. Acad. Sci. USA. 89:8759-8763.

Barbieri, L., M.G. Battelli, and F. Stirpe. 1993. Ribosome-inactivating proteins from plants. Biochim Biophys Acta. 1154:237-282.

Baulcombe, D. 1994. Novel strategies for engineering virus resistance in plants. Current Opinion in BioTechnology. 5:117-124.

Benfey, P., L. Ren, and N.-H. Chua. 1989. The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J. 8:2195-2202.

Bennett, A., and K. Osteryoung. 1991. Protein transport and targeting within the endomembrane system of plants. In Plant Genetic Engineering. Vol. 1. D. Grierson, editor. Blackie publishing, London. 199-237.

Benvenuto, E., R.J. Ordas, R. Tavazza, G. Ancora, S. Biocca, A. Cattaneo, and P. Galeffi. 1991. 'Phytoantibodies': a general vector for the expression of immunoglobulin domains in transgenic plants. Plant Mol Biol. 17:865-74.

Bird, R.E., K.D. Hardman, J.W. Jacobson, S. Johnson, B.M. Kaufmann, S.-M. Lee, T. Lee, H.S. Pope, G.S. Riordan, and M. Whitlow. 1988. Single-chain antigen-binding proteins. Science. 242:423-426.

Bjorck, L., P. Akesson, M. Bohus, J. Trojnar, M. Abrahamson, I. Olafsson, and A. Grubb. 1989. Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor. Nature. 337:385-6.

Bjorck, L., A. Grubb, and L. Kjellen. 1990. Cystatin C, a human proteinase inhibitor, blocks replication of herpes simplex virus. Nature. 337:385-386.

Caddick, M., A. Greenland, I. Jepson, K. Krause, N. Qu, K. Riddell, M. Salter, W. Schuch, U. Sonnewald, and A. Tomsett. 1998. An ethanol inducible gene switch for plants used to manipulate carbon metabolism. Nat. Biotechnol. 16:177-180.

Carter, P., and A. Merchant. 1997. Engineering antibodies for imaging and therapy. Curr Opin Biotechnol. 8:449-454.

Carter, P., J. Ridgway, and Z. Zhu. 1995. Toward the production of bispecific antibody fragments for clinical applications. J Hematother. 4:463-470.

Chen, S.Y., Y. Khouri, J. Bagley, and W.A. Marasco. 1994. Combined intra- and extracellular immunisation against human immunodeficiency virus type 1 infection with a human anti-gp 120 antibody. Proc. Natl. Acad. Sci USA. 91:5932-5936.

Chowrira, G., V. Akella, and P. Lurquin. 1995. Electroporation-mediated gene transfer into intact nodal meristems in planta. Generating transgenic plants without in vitro tissue culture. Mol Biotechnol. 3:17-23.

Comai, L., P. Moran, and D. Maslyar. 1990. Novel and useful properties of a chimeric plant promoter combining CaMV 35S and MAS elements. Plant Mol. Biol. 15:373-381.

De Neve, M., M. De Loose, A. Jacobs, H. Van Houdt, B. Kaluza, U. Weidle, M. Van Montagu, and A. Depicker. 1993. Assembly of an antibody and its derived antibody fragment in Nicotiana and Arabidopsis. Transgenic Res. 2:227-37.

Dempsey, D., H. Silva, and D. Klessig. 1998. Engineering disease and pest resistance in plants. Trends Microbiol. 6:54-61.

D'Halluin, K., E. Bonne, M. Bossut, M. De Beuckeleer, and J. Leemans. 1992. Transgenic maize plants by tissue electroporation. Plant Cell. 4:1495-1505.

Duan, L., O. Bagasra, M.A. Laughlin, J.W. Oakes, and R.J. Pomerantz. 1994. Potent inhibition of human immunodeficiency virus type 1 replication by an intracellular anti-Rev single chain antibody. Proc. Natl. Acad. Sci USA. 91:5075-5079.

Düring, K., S. Hippe, F. Kreuzaler, and J. Schell. 1990. Synthesis and selfassembly of a functional monoclonal antibody in transgenic Nicotiana tabacum. Plant Mol. Biol. 15:281-293.

Eiklid, K., S. Olsnes, and A. Pihl. 1980. Entry of lethal doses of abrin, ricin and modeccin into the cytosol of HeLa cells. Exp Cell Res. 126:321-6.

Endo, Y., and K. Tsurugi. 1987. RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J Biol Chem. 262:8128-30.

Endo, Y., and K. Tsurugi. 1988. The RNA N-glycosidase activity of ricin A-chain. Nucleic Acids Symp Ser. 19:139-42.

Falk, B.W., and G. Bruening. 1994. Will transgenic crops generate new viruses and new diseases. Science. 263:1395-1396.

Fiedler, U., J. Phillips, O. Artsaenko, and U. Conrad. 1997. Optimisation of scFv antibody production in transgenic plants. Immunotechnology. 3:205-216.

Fischer, R.S., A. Schillberg, Y.-D. Stierhof, and F. Kreuzaler. 1999. Production, characterisation and molecular cloning of Tobacco Mosaic Virus (TMV)-specific neutralizing monoclonal antibodies with different epitope specificities. Molecular Immunology.

Florack, D., W. Dirkse, B. Visser, F. Heidekamp, and W. Stiekema. 1994. Expression of biologically active hordothionins in tobacco. Effects of pre- and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting. Plant Mol Biol. 24:83-96.

Friedler, A., N. Zakai, O. Karni, Y. Broder, L. Baraz, M. Kotler, A. Loyter, and C. Gilon. 1998. Backbone cyclic peptide, which mimics the nuclear localisation signal of human immunodeficiency virus type 1 matrix protein, inhibits nuclear import and virus production in nondividing cells. Biochemistry. 37:5616-5622.

Furth, P. 1997. Gene transfer by biolistic process. Mol Biotechnol. 7:139-143.

Gadani, F., M. Mansky, R. Medici, W.A. Miller, and J.H. Hill. 1990. Genetic engineering of plants for virus resistance. Archives of Virology. 115:1-21.

Gerber, L.D., K. Kodukula, and S. Udenfriend. 1992. Phosphatidylinositol glycan (PI-G) anchored membrane proteins. Amino acid requirements adjacent to the site of cleavage and PI-G attachment in the COOH-terminal signal peptide. J Biol Chem. 267:12168-73.

Girbes, T., J. Ferreras, R. Iglesias, L. Citores, C. De Torre, M. Carbajales, P. Jimenez, F. De Benito, and R. Munoz. 1996. Recent advances in the uses and applications of ribosome-inactivating proteins from plants. Cell Mol Biol (Noisy-le-grand). 42:461-471.

Gross, G., and Z. Eshhar. 1992. Endowing T cells with antibody specificity using chimeric T cell receptors. Faseb J. 6:3370-8.

Ham, P.J., C. Albuquerque, B. Smithies, R. Chalk, S. Klager, and H. Hagen. 1994. Antibacterial peptides in insect vectors of tropical parasitic disease. Ciba Found Symp. 186:140-51.

Harrison, B.D., M.-A. Mayo, and D.C. Baulcombe. 1987. Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. Nature. 328:799-802.

Hartley, M.R., J.A. Chaddock, and M.S. Bonness. 1996. The structure and function of ribosome inactivating proteins. Trends in Plant Science. 1:254-260.

Hayakawa, Y. 1991. Structure of a growth-blocking peptide present in parasitzed insect hemolymph. J Biol Chem. 266:7982-7984.

Hiatt, A., R. Cafferkey, and K. Bowdish. 1989. Production of antibodies in plants. Nature. 342:469-470.

Kapila, J., R. De Rycke, M. Van Montagu, and G. Angenon. 1997. An Agrobacterium mediated transient gene expression system for intact leaves. Plant Sci. 122:101-108.

Kim, P., F. Janiak-Spens, W. Trimble, B. Leber, and D. Andrews. 1997. Evidence for multiple mechanisms for membrane binding and integration via carboxyl-terminal insertion sequences. Biochemistry. 36:8873-8882.

Klein, T., and S. Fitzpatrick-McElligott. 1993. Particle bombardment: a universal approach for genetransfer to cells and tissues. Curr Opin Biotechnol. 4:583-590.

Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 256:495-497.

Lambert, S., and V. Bennett. 1993. From anemia to cerebellar dysfunction. A review of the ankyrin gene family. Eur J Biochem. 211:1-6.

LaVallie, E., E. DiBlasio, S. Kovacic, K. Grant, P. Schendel, and J. McCoy. 1993. A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. Biotechnology (N Y). 11:187-193.

Leland, P., L. Schultz, B. Kim, and R. Raines. 1998. Ribonuclease A variants with potent cytotoxic activity. Proc Natl Acad Sci U S A. 95:10407-10412.

Madshus, I., and H. Stenmark. 1992. Entry of ADP-ribosylating toxins into cells. Curr Top Microbiol Immunol. 175:1-26.

Marasco, W.A., W.A. Haseltine, and S.Y. Chen. 1993. Design, intracellular expression, and activity of a human anti-human immunodeficiency virus type 1 gp 120 single-chain antibody. Proc. Natl. Acad. Sci USA. 90:7889-7893.

Mitra, A., and A. Gynheung. 1989. Three distinct regulatory elements comprise the upstream promoter of the nopaline synthase gene. Mol. Gen. Genet. 215:294-299.

Montanaro, L., S. Sperti, A. Mattioli, G. Testoni, and F. Stirpe. 1975. Inhibition by ricin of protein synthesis in vitro. Inhibition of the binding of elongation factor 2 and of adenosine diphosphate-ribosylated elongation factor 2 to ribosomes. Biochem J. 146:127-31.

Namba, S., K. Ling, C. Gonsalves, D. Gonsalves, and J.L. Slightom. 1991. Expression of the gene encoding the coat protein of cucumber mosaic virus (CMV) strain WL appears to provide protection to sugarbeet plants against infection by several different strains. Gene. 107:181-188.

Ni, M., D. Cui, J. Einstein, S. Narasimhulu, C.E. Vergara, and S.B. Gelvin. 1995. Strength and tissue specificity of chimeric promoters derived from octopine and nopaline synthase genes. Plant J. 7:661-676.

Owen, M., A. Gandecha, B. Cockburn, and G. Whitelam. 1992. Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco. Bio/Technology. 10:790-794.

Palukaitis, P., and M.J. Roossinck. 1996. Spontaneous change of a benign satellite RNA of cucumber mosaic virus to a pathogenic variant. Nature Biotechnology. 14:1264-1268.

Peters, L.L., and S.E. Lux. 1993. Ankyrins: structure and function in normal cells and hereditary spherocytes. Semin Hematol. 30:85-118.

Plückthun, A. 1991. Antibody engineering. Current Opinion in Biotechnology. 2:238-246.

Pluckthun, A., and P. Pack. 1997. New protein engineering approaches to multivalent and bispecific antibody fragments. Immunotechnology. 3:83-105.

Poljak, R.J. 1994. Production and structure of diabodies. Structure. 2:1121-3.

Rose, M., and G. Fink. 1987. KAR1, a gene required for function of both intranuclear and extranuclear microtubules in yeast. Cell. 48:1047-1060.

Sanford, J.C., E.D. Wolf, and N.K. Allen. 1990. Method for transporting substances into living cells and apparatus therefore. Patent #4,945,050. Cornell Research Foundation Inc, Ithaca, NY.

Sangster, B. 1997. Identification of cytotoxic peptide as possible mechanism for neurotoxicity of HIV viral envelope and AIDS pathogenesis. Med Hypotheses. 48:463-8.

Schouten, A., J. Roosien, J.M. de Boer, A. Wilmink, M.-N. Rosso, D. Bosch, W.J. Stiekema, F.J. Gommers, J. Bakker, and A. Schots. 1997. Improving scFv antibody expression levels in the plant cytosol. FEBS letters. 415:235-241.

Silburn, K., D. McPhee, A. Maerz, P. Poumbourios, R. Whittaker, A. Kirkpatrick, W. Reilly, M. Manthey, and C. Curtain. 1998. Efficacy of fusion peptide homologs in blocking cell lysis and HIV-induced fusion. AIDS Res Hum Retroviruses. 14:385-392.

Smith, D., and K. Johnson. 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene. 67:31-40.

Spörlein, B., and H.-U. Koop. 1991. Lipofectin: direct gene transfer to higher plants using cationic liposomes. Theor. Appl. Genet. 83:1-5.

Tang, Y., N. Jiang, C. Parakh, and D. Hilvert. 1996. Selection of linkers for a catalytic single-chain antibody using phage display technology. J Biol Chem. 271:15682-6.

Tavladoraki, P., E. Benvenuto, S. Trinca, D. DeMartinis, and P. Galeffi. 1993. Transgenic plants expressing a functional scFv antibody are protected from virus attack. Nature. 366:469-472.

Tedder, T.F., and P. Engel. 1994. CD20: a regulator of cell-cycle progression of B lymphocytes. Immunol Today. 15:450-4.

Turner, D.J., M.A. Ritter, and A.J. George. 1997. Importance of the linker in expression of single-chain Fv antibody fragments: optimisation of peptide sequence using phage display technology. J Immunol Methods. 205:43-54.

Turpen, T.H., A.M. Turpen, N. Weinzettl, M.H. Kumagai, and W.O. Dawson. 1993. Transfection of whole plants from wounds inoculated wtih Agrobacterium tumefaciens containing cDNA of Tobacco Mosaic Virus. J. Virol. Meth. 42:227-240.

von Heijne, G. 1985. Signal sequences. The limits of variation. J Mol Biol 1985. 184:99-105.

Voss, A., M. Niersbach, R. Hain, H.J. Hirsch, Y.C. Liao, F. Kreuzaler, and R. Fischer. 1995. Reduced virus infectivity in N. tabacum secreting a TMV-specific full size antibody. Mol. Breeding. 1:39-50.

White, F.F. 1992. Vectors for gene transfer in higher plants. In Transgenic Plants. Vol. 1. Academic Press. 15-47.

Wilson, T.M.A. 1993. Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms. Proc. Natl. Acad. Sci. USA. 90:3134-3141.

Winter, G., A.D. Griffiths, R.E. Hawkins, and H.R. Hoogenboom. 1994. Making antibodies by phage display technology. Annu Rev Immunol. 12:433-55.

Winter, G., and C. Milstein. 1991. Man made antibodies. Nature. 349:293-299.

Wright, M.D., and M.G. Tomlinson. 1994. The ins and outs of the transmembrane 4 superfamily. Immunol Today. 15:588-94.

Zimmermann, S., S. Schillberg, Y.C. Liao, and R. Fischer. 1998. Intracellular expression of a TMV-specific single chain Fv fragment leads to improved virus resistance in Nicotiana tabacum. Molecular Breeding. 4:369-379.